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University of Alberta

INFLUENCES OF ESTROGEN AND GENDER ON CENTRAL AUTONOMIC  
RESPONSES TO RESTRAINT

By

Heather Edgell



A thesis submitted to the Faculty of Graduate Studies and Research in partial  
fulfillment of the requirements for the degree of Master of Science

Department of Cell Biology

Edmonton, Alberta  
Fall, 2001





**University of Alberta**

**Faculty of Graduate Studies and Research**

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Influences of Estrogen and Gender on Central Autonomic Responses to Restraint** by Heather Edgell in partial fulfillment of the requirements for the degree of Master of Science.





## **Abstract**

The loss of estrogen at menopause increases the risk of cardiovascular disease in women, and estrogen replacement therapy is used for the prevention of cardiovascular disease in postmenopausal women. It is not clear how estrogen and gender affect central autonomic responses to psychological (restraint) stress. To test the hypothesis that estrogen attenuates central sympathetic responses during restraint in rats, we measured mean arterial pressure (MAP), heart rate (HR), and central nitric oxide (NO) production in response to restraint in estrogen or vehicle-treated ovariectomized females, diestrus females and males. Attenuation of sympathetic output was associated with estrogen treatment, and attenuated baroreceptor reflexes were found in ovariectomized females and males in comparison to intact females. Increased NO production was associated with estrogen treatment, ovariectomy, and gender. The effects of estrogen treatment on central pathways may be responsible for the cardioprotective effects seen with estrogen replacement and may be mediated by central NO.



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## LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin hormone
AVP	arginine vasopressin
BP	blood pressure
BSA	bovine serum albumin
CNS	central nervous system
CRF	corticotropin releasing factor
cGMP	cyclic guanosine monophosphate
cRNA	complementary ribonucleic acid
DAB	diaminobenzidine tetrahydrochloride
DMN <sub>x</sub>	dorsal motor nucleus of the vagus
DNA	deoxyribonucleic acid
dp	dorsal parvocellular subdivision of middle PVN
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERE	estrogen response element
ERT	estrogen replacement therapy
FSH	follicle stimulating hormone
GC	glucocorticoids
GnRH	gonadotropin releasing hormone
HPA	hypothalamic-pituitary-adrenal



HR	heart rate
hsp90	heat shock protein 90
IML	intermediolateral cell column of the spinal cord
iNOS	inducible nitric oxide synthase
L-DOPA	3,4-dihydroxyphenylalanine
LC	locus coeruleus
LH	luteinizing hormone
L-NAME	N <sup>G</sup> -nitro-L-arginine methyl ester
L-NMA	N <sup>ω</sup> -methyl-L-arginine
LPS	lipopolysaccharide
LTE	long-term estrogen treated ovariectomized rats
LTV	long-term vehicle treated ovariectomized rats
MAP	mean arterial blood pressure
mpd	dorsal medial parvocellular subdivision of the middle PVN
mpv	ventral medial parvocellular subdivision of the middle PVN
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NADPHd	nicotinamide adenine dinucleotide phosphate diaphorase
nNOS	neuronal nitric oxide
NO	nitric oxide





NOS	nitric oxide synthase
NTS	nucleus of the tractus solitarius
OVX	ovariectomized
OVX-E	estrogen-treated ovariectomized
OVX-V	vehicle-treated ovariectomized
OXY	oxytocin
PBS	phosphate-buffered saline
pm	posterior magnocellular subdivision of the middle PVN
PNMT	phenylethanolamine N-methyltransferase
PVN	paraventricular nucleus
RNase	ribonuclease
SEM	standard error of the mean
SON	supraoptic nucleus
SSC	standard saline citrate
TH	tyrosine hydroxylase
VLM	ventrolateral medulla



## LIST OF SYMBOLS

$*$ , @, #, +, %, all denote significant difference,  $p < 0.05$

↓, time at which animals were put into the restraint tube

⬇, time at which animals were taken out of the restraint tube





# **CHAPTER 1**

## **INTRODUCTION**



## **1.1. Overview**

Postmenopausal women are often treated with estrogen replacement therapy (ERT) for the alleviation of menopausal symptoms. More recently ERT has been found to be effective in attenuating bone loss due to osteoporosis, and reducing the risk of cardiovascular disease in postmenopausal women (Reviewed in Belchetz, 1994 and Schapner et al., 2000). Central pathways which estrogen can affect are those that regulate the hypothalamo-pituitary-adrenal (HPA) axis and sympathetic output. These pathways are involved in the physiological response to stress. Nitric oxide (NO) is a gaseous unconventional neurotransmitter that acts as a peripheral vasodilator, and is released in the brain during stress to restore homeostasis. Effects of estrogen on central NO may mediate the cardiovascular benefit associated with ERT. While it is known that estrogen affects the brain, it is not yet known how estrogen replacement in ovariectomized female rats affects the HPA axis, sympathetic output, and central NO production during restraint stress. The sexual dimorphic response to restraint in all of the above pathways is also not well characterized.

## **1.2. Hypothalamic-pituitary-adrenal (HPA) axis and sympathetic output**

The body has many ways of responding to stressful situations. These reactions are centrally mediated by particular areas of the brain including the



paraventricular nucleus of the hypothalamus (PVN), nucleus of the tractus solitarius (NTS), ventrolateral medulla (VLM) and the locus coeruleus (LC) (Reviewed in Stratakis et al., 1995). The paraventricular nucleus (PVN) of the hypothalamus can be subdivided into subdivisions in rostral, middle, and caudal segments. The middle segment of the PVN can be further subdivided into the posterior magnocellular (pm) division, the dorsal parvocellular (dp) division, the dorsal medial parvocellular (mpd) division, and the ventral medial parvocellular (mpv) division (Figure 1). Their functions will be discussed below.

The paraventricular nucleus of the hypothalamus (PVN) is important for autonomic regulation. The magnocellular neurons have projections to the posterior pituitary, and are responsible for the production of arginine vasopressin (AVP) and oxytocin (OXY; Reviewed in Sawchenko et al., 1996). Generally, these hormones are involved in water regulation and sexual behavior. More specifically, AVP is known to promote resorption of water and constriction of blood vessels. OXY is involved in parturition and milk ejection (Reviewed in Sawchenko et al., 1996).

One of the major roles of the mpd division is the production of CRF in neurons that project to the anterior pituitary via the median eminence and activates the hypothalamic-pituitary-adrenal (HPA) axis (Reviewed in Sawchenko et al., 1996). CRF stimulates the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary into the blood stream to induce the release of glucocorticoids (GC) from the adrenal cortex (Chapter 77 in



Guyton, 1991) that act to suppress the immune system and act as a negative feedback upon CRF release (Chapter 77 in Guyton, 1991). This negative feedback is mediated via GC receptors in the hippocampus which has projections to many brain areas including the PVN (Ferrini et al, 1999).

The dp and mpv divisions contain neurons that are partially responsible for sympathetic output via projections to other brain areas including the nucleus of the tractus solitarius (NTS), ventrolateral medulla (VLM), the intermediolateral cell column (IML) of the spinal cord, and the locus coeruleus (LC; Loewy, 1981; Figure 2). The VLM and NTS are responsible for integrating cardiovascular functions and vasomotor tone (Kalia et al., 1984; Calaresu et al., 1984). Projections from the VLM and PVN descend upon the IML, the site of sympathetic preganglionic neurons (Reviewed in Calaresu et al., 1984). The LC is made up of catecholaminergic neurons (Dahlstrom and Fuxe, 1964) with axons that join with other catecholaminergic axons from the pons and medulla to form an ascending pathway terminating on the PVN among other areas (Loizou, 1969). All the previously named areas are important in regulating sympathetic output. The CNS can receive peripheral stimuli such as stress through these pathways (Palkovits et al., 1995). For example, changes in blood volume or pressure due to stress can change the firing rate of LC neurons (Elam et al., 1984; Julien et al., 1995).

Upon increased BP, baroreceptors found in the aortic arch and carotid sinus increase firing, causing decreased sympathetic output from the CNS to





the heart and blood vessels (Green and Heffron, 1968; Koizumi et al., 1971). Blood pressure and heart rate together are suitable indicators of changed sympathetic output via the baroreceptor reflex, a centrally mediated sympathetic reflex that effects both measurements (Kumada et al., 1990). Input from the arterial baroreceptors affects the activity of various CNS areas involved in controlling the circulation, including the NTS and VLM in the medulla (Brattstrom et al., 1992; Gebber and Barman, 1985), and the PVN of the hypothalamus (Cechetto and Calaresu, 1983). The activation of aortic baroreceptors inhibits the LC, which is also involved in the regulation of blood pressure (Murase et al., 1994). The final effect is to restore homeostasis to the cardiovascular system by changing MAP and HR. The baroreceptor reflex is a centrally mediated reflex that can be used as a tool to study central cardiovascular control mechanisms, in particular the sympathetic nervous system (Kumada et al., 1990).

### **1.3. Stress**

#### **1.3.1. Effects of Stress on the HPA axis and Sympathetic Output**

Complex neuronal circuitry, in particular that of the sympathoadrenal system and HPA axis, has evolved in animals to help respond to stress (Chrousos and Gold, 1995). The term “stress” has been defined as some factor or state of being that disrupts normal physiological and psychological function to produce broad biological effects (Kopin, 1995). There are many types of



stress, including physiological and psychological stress. Experimental examples of each would be injections of lipopolysaccharide (LPS), a major component of the bacterial cell wall, to imitate biological infection (physiological stress), or immobilization stress such as restraint (psychological stress).

Exposure to stressors activates both the sympathetic nervous system and the HPA axis, resulting in the release of CRF from the PVN, ACTH from the pituitary and catecholamines from the adrenal cortex (Kvetnansky et al., 1995; Pacak et al., 1995; Givalois et al., 2000). Blood pressure and cardiac output both increase in people during mental stress (Herd, 1991). This response can be described as part of the fight-or-flight response to stress, causing increased sympathetic output, and increased glucocorticoids to the adrenal glands (Chapter 60 in Guyton, 1991). Restraint stress in males has been shown to increase both CRF mRNA levels and the number of activated neurons in the PVN, thus increasing HPA axis activity (Mansi et al., 1998; Duncko et al., 2001; Imaki et al., 1998). Similarly, a 3 day chronic stress regimen led to increased plasma corticosterone levels in both females and males, with a higher level in female rats (Duncko et al., 2001). In contrast, other data show that 15 minutes of psychological stress did not change CRH mRNA levels in the PVN (Makino et al., 1999). Since the animals were sacrificed 1.5 and 3-hr after cessation of stress, the effects could have been missed since 4 hours is typically needed for the development of mRNA.



The locus coeruleus (LC) is activated during times of stress (Valentino et al., 1993). The LC is also known to mediate the effects of stress via tyrosine hydroxylase (TH) activity and catecholamine release (Korf et al., 1973). Tyrosine hydroxylase (TH) is the rate-limiting enzyme involved in catecholamine biosynthesis; it catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), which then is decarboxylated to form dopamine. The dopamine can then be further processed to become norepinephrine or epinephrine (Reviewed in Sabban et al., 1995). Immobilization stress has been shown to increase TH mRNA levels in the LC and adrenal medulla (Serova et al., 1999; Nankova et al., 1999; Smith et al., 1991; Sands et al., 2000) and increase adrenal TH immunoreactivity (Hoeldtke et al., 1974). On the other hand, chronic mild stress (6 weeks of alternating mild stressors) decreases TH mRNA in the LC (Duncko et al., 2001).

The HPA axis and sympathetic output are known to affect one another. Studies of the interactions between the HPA axis and the sympathoadrenal system have shown that glucocorticoids can affect catecholamine synthesis during basal conditions and during stress (Kvetnensky et al., 1995). It has been seen that 30 minutes of restraint stress increased plasma corticosterone levels while decreasing hypothalamic norepinephrine levels (Keim and Sigg, 1976). In contrast, the rise of glucocorticoids seen with immobilization stress has been shown to increase gene expression of TH and the last enzyme activated in catecholamine biosynthesis (PNMT; Sabban et al., 1995). It has





also been shown that CRF can activate noradrenergic neurons of the LC (Schulz and Lehnert, 1996), therefore showing an interaction between HPA axis activity and sympathetic output.

## **1.4. Physiological Effects of Estrogen**

Endogenous estrogen cyclically circulates through the female body, and is produced in the ovaries. The typical rat estrous cycle lasts approximately 4 days. The stages of estrous consist of estrus, followed by metestrus, diestrus, and proestrus. The plasma estradiol concentrations of female rats throughout the estrous cycle are  $17 \pm 2$  pg/ml to  $21 \pm 2$  pg/ml between estrus and metestrus,  $37 \pm 5$  pg/ml during late metestrus, and a peak value of  $88 \pm 2$  pg/ml during proestrus (Butcher et al., 1974). In contrast, Leipheimer et al. (1984) found estrogen plasma concentration during diestrus to be  $18.2 \pm 1.2$  pg/ml, a much lower value than one interpolated from the previous data (estimated at 40-60 pg/ml).

### **1.4.1. Peripheral Effects**

Estrogen is an important hormone that is responsible for the development of secondary sex characteristics in females (i.e. growth of the uterus, external sex organs, and breasts). These effects are elicited via binding to cellular receptors in the target organs (Chapter 81 in Guyton, 1991).



Estrogen is also important in regulation of other sex hormones in the body. In small amounts, estrogen inhibits the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the anterior pituitary, whereas estrogen in large amounts seems to stimulate LH and FSH secretion (Chapter 81 in Guyton, 1991). During proestrus, the peak concentrations of LH and FSH immediately followed the estradiol peak (Butcher et al., 1974). FSH and LH are released at highest concentration immediately before ovulation, then the corpus luteum in the ovaries releases high levels of estrogen and progesterone. High progesterone levels inhibit the production of FSH and LH, thus opposing the actions of estrogen to reduce the secretion of FSH and LH (Chapter 81 in Guyton, 1991).

#### **1.4.2. Autonomic Effects**

Estrogen treatment has been shown to decrease sympathetic activity. Premenopausal and women undergoing estrogen replacement have a reduced risk of cardiovascular disease than untreated postmenopausal women or men (Contreras and Parra, 2000). Estrogen can directly inhibit vascular smooth muscle contraction (Reviewed in Ruehlmann and Mann, 2000). For example, after mental arithmetic stress, postmenopausal women showed higher vasoconstriction than premenopausal women or those with estrogen replacement (Sung et al., 1999). As well, 17- $\beta$  estradiol has been shown to reduce pressure from the left ventricle of isolated rabbit hearts (Raddino et al.,



1986), and to elicit relaxation of rat thoracic aorta (Conde et al., 2000; Browne et al., 1999). In both male and ovariectomized female rats given estrogen, baroreceptor reflexes are enhanced compared to respective controls so that parasympathetic nerve activity is increased and sympathetic nerve activity is decreased (He et al., 1999; Saleh and Connell, 1998; Saleh and Connell, 2000). The animals showed augmented reflex bradycardia in response to increased MAP. Others have found that females have a less sensitive baroreceptor reflex in comparison to males. At the same time, OVX-E rats have a higher baroreceptor reflex in comparison to sham-operated females, but not different than age-matched males (El-Mas et al., 1997). It was also found that in OVX-E rats, an injection of an estrogen receptor antagonist, ICI 162 780, into the intrathecal space of the spinal cord blocked changes in parasympathetic and sympathetic nerve activities after blood pressure changes. Thus, estrogen can modulate the baroreflex via the activation of central estrogen receptors (Saleh and Connell, 2000).

## **1.5. Estrogen receptors**

### **1.5.1. Genomic and non-genomic effects**

Estrogen's effects can be initiated by either genomic or non-genomic means. The genomic model for estrogen action suggests that the estrogen crosses the plasma membrane, enters the cytoplasm, binds to and activates intracellular receptors. These activated receptors bind to the DNA and act as



transcription factors for other genes (Moore and Evans, 1999). The more recent non-genomic model suggests that estrogen binds to membrane bound receptors to elicit signaling pathways (Reviewed in McEwan, 1991 and Moore and Evans, 1999). The latter model could be responsible for rapid steroid responses that cannot be explained by the genomic model that predicts a longer time period between steroid exposure and responses. (McEwan and Alves, 1999).

### **1.5.2. Receptor types and localization**

There are 4 types of hormone receptors, an intracellular ligand dependent transcription factor (genomic regulation), and 3 types of membrane-associated receptors (non-genomic regulation). These membrane-associated receptors are ligand-gated ion channels, enzyme-linked receptors, and G-protein coupled receptors (Reviewed in Moore and Evans, 1999). The membrane ER is structurally similar to the intracellular ER (Pappas et al., 1995). Therefore, a membrane ER may originate from the same transcript as intracellular ER (Razandi et al., 1999).

Two known estrogen receptor isoforms, ER $\alpha$  and ER $\beta$ , are located throughout the body and brain. ER $\alpha$  is important in sexual behavior and reproductive functions, whereas ER $\beta$  influences autonomic regulation, such as sympathetic and HPA axis activity (Laflamme et al., 1998). ER $\beta$  receptors have been localized in areas known to influence HPA axis activity (PVN) and





sympathetic activity (PVN, NTS, VLM, LC; Shughrue et al., 1997). ER $\alpha$  is necessary for the estrogen-mediated regulation of reproduction, including sexual behavior (Reviewed in Rissman et al., 1999). They are found in the pituitary, adrenal glands, brainstem and forebrain particularly the hypothalamus among other areas (Boers et al., 1999; Laflamme et al., 1998). Gonadotropin releasing hormone (GnRH) neurons also express functional ERs (Prevot et al., 1999).

As mentioned, ER $\beta$  is important in autonomic regulation. ER $\beta$  has been found in the olfactory bulbs, cerebellum, cerebral cortex, with moderate to high expression in prostate, lung, and bladder, and high expression in brain, ovary, testis, and uterus (Simonian and Herbison, 1997; Kuiper et al., 1996; Laflamme et al., 1998; Reviewed in Giguere et al., 1998). ER $\beta$  is found in some magnocellular neurons in the PVN and supraoptic nucleus (SON) of the hypothalamus (Alves et al., 1998; Hrabovszky et al., 1998). Factors such as gonadal state, or the stage of estrous of the animal, appear to affect both ER $\alpha$  and ER $\beta$  protein levels in the brain (Shughrue et al., 1992; Osterlund et al., 1998). It is now known that at least two isoforms of ER $\beta$  exist, ER $\beta$ 1 (original) and ER $\beta$ 2 (Petersen et al., 1998), and that estrogen receptors can act in the form of homodimers or heterodimers (ER $\beta$  and ER $\beta$ 2 with each other and with ER $\alpha$ )(Hanstein et al., 1999).

ER $\beta$ 2 binds to the estrogen response element (ERE) of DNA, and acts as a negative regulator, suppressing ER $\alpha$  and ER $\beta$ 1-mediated transcriptional activation (Reviewed in McEwan and Alves, 1999). ERs can also affect



transcription via the AP-1 response element of genes (Uht et al., 1997). For ER $\beta$ 2 to become activated, 17 $\beta$ -estradiol concentration must be 100-1000 fold higher than that for activation of either ER $\alpha$  or ER $\beta$ 1 (Hanstein et al., 1999). All three isoforms are found in equal amounts in the ovary, prostate and pituitary, but ER $\beta$ 1 was 2-6 times more abundant than ER $\beta$ 2 in the hypothalamus and hippocampus (Petersen et al., 1998). Thus, in order for negative regulation to occur, estrogen levels must be extremely high.

### **1.5.3. Effects of Estrogen on the HPA axis and Sympathetic Output During Stress**

There are opposing data concerning estrogen's effects upon the HPA axis. Some data have shown that ACTH and corticosterone plasma levels increase after 5-second footshock stress in estrogen-treated ovariectomized rats (OVX-E)(Burgess and Handa, 1992), whereas more recent data have shown that ether stress decreases ACTH and corticosterone plasma levels in old estrogen treated male rats (Ferrini et al., 1999). Similarly to the former data, upon 20 minute restraint stress cycling female rats in proestrus (phase of estrus with the highest level of endogenous estrogen) have higher ACTH and corticosterone in plasma than rats in diestrus or estrus (Viau and Meaney, 1991). Discrepancies could be due to the different stressors used, the methods of plasma recovery, and/or removal of ovaries and the hormones produced there. The response to stress can also depend on many other factors including



duration of restraint, sex, age, handling, housing conditions, light-dark cycle and estrus phase (Keim and Sigg, 1976).

The female hypothalamus is more predisposed to changing corticosteroid receptor expression in response to restraint stress than males, but the elimination of female steroids (via ovariectomy) did not lead to the male pattern of receptor expression (Karandrea et al., 2000). Thus, a distinct male/female neuroendocrine axis in response to stress exists. OVX-E rats have 20% fewer GC receptors than intact males in the pituitary. Thus, lower numbers of receptors in OVX-E rats may reduce negative feedback at the level of the pituitary, or increase HPA axis activity (Turner et al., 1990). Currently, there is little known about the effects of estrogen on sympathetic output during stress, and will be explored in this thesis.

## **1.6. Nitric oxide (NO)**

In 1988, endothelium derived neurotrophic factor (EDNF), originally known for its effects as a vasodilator (Reviewed in Garthwaite et al., 1988), was found to be an intercellular messenger in the brain (Garthwaite et al., 1988). EDNF was later determined to be nitric oxide (NO; Palmer et al., 1987). NO acts as a gaseous, lipophilic, unconventional neurotransmitter (Reviewed in Krukoff, 1999) that diffuses in all directions equally from a point source (Wood and Garthwaite, 1994).



There are 3 known isoforms of NOS, two constitutive forms, endothelial NOS (eNOS) and neuronal NOS (nNOS), and one inducible form (iNOS). The constitutive isoforms are calcium-dependent, and require calmodulin binding upon cellular calcium influx (Brenman and Bredt, 1997). This calcium influx is due to the activation of NMDA receptors by glutamate, leading to activation of constitutive NOS via the binding of calmodulin (Figure 3; Garthwaite et al., 1989, Garthwaite et al., 1988; Luo and Vincent, 1994; Reviewed in Krukoff, 1999). The inducible form is calcium independent because calmodulin is permanently bound (Mayer and Hemmens, 1997), and is usually activated by cytokines (Damoulis and Hauschka, 1994). Nitric oxide synthase (NOS) is responsible for converting L-arginine into NO and L-citrulline (Figure 3). Responses are elicited via the stimulation of cGMP production by binding the heme group of soluble guanylyl cyclase (Bredt and Snyder, 1989; Southam and Garthwaite, 1993; Mayer and Hemmens, 1997; Zhao et al., 1999). The cGMP then elicits cellular responses via cGMP-dependent protein kinases, cGMP regulated ion channels, and/or cGMP regulated cyclic nucleotide phosphodiesterases (Lincoln and Cornwell, 1993). NO is degraded through reactions with oxygen to produce nitrates and nitrites (Salter et al., 1996). Activation of soluble guanylyl cyclase is thought to produce vascular smooth muscle relaxation (Papapetropoulos et al., 1994).

Administration of L-NMA, an inhibitor of NOS, raises arterial blood pressure and enhances central sympathetic outflow (Togashi et al., 1992). When NOC 18, an NO donor, was injected into the rostral ventrolateral





medulla (brain stem) of rats, the mean arterial blood pressure (MAP) was significantly decreased. When L-NAME, a NOS inhibitor, was injected MAP increased (Kagiyama et al., 1997). Thus, NO can act in the central nervous system to reduce vascular sympathetic tone.

### **1.6.1. NO production is activated by stress**

Stress has been shown to increase NO production to restore homeostasis to a physiological system (Krukoff, 1999). Acute restraint stress has been shown to increase the activity of iNOS, and increases the expression of iNOS in the cortex of male rats (Madriral et al., 2001). Amir et al. (1997) showed that immobilization stress activates c-fos expression (immediate early gene) in the PVN, and NO is involved in this activation. Even slight stressors such as a change in environment or darkness can significantly increase the number of NADPHd stained (nNOS containing) neurons in the PVN (Sanchez et al., 1999). Immobilization stress upregulates nNOS mRNA expression in anterior pituitary, adrenal cortex, and the PVN (Kishimoto et al., 1996). Upon increasing levels of psychological stress, there is activation of NO-producing neurons in the PVN, NTS and parts of the VLM (Krukoff and Khalili, 1997). The above suggests that NO plays a role in stress-induced activation of the HPA axis and/or sympathetic output.



### **1.6.2. Involvement of NO in HPA axis and sympathetic output**

NO is present in neuronal centers responsible for both the HPA axis and sympathetic output. Histochemical mapping of nNOS (NADPHd staining) in the rat brain localized nNOS to the PVN (important for both the HPA axis and sympathetic output), NTS, VLM, dorsal motor nucleus of the vagus (DMNx; parasympathetic afferent), and the IML cell column (important in regulating sympathetic activity; Vincent and Kimura, 1992). Messenger RNA of all 3 isoforms has been found in the hypothalamus of female rats, where nNOS is the major form present with less eNOS and no observed iNOS (Bhat et al., 1996). When NO blockers are microinjected into the NTS of rats, increases in arterial pressure and renal sympathetic nerve activity are seen, as is an increased heart rate upon baroreceptor and vagus removal (Harada et al., 1993). In the PVN, nNOS expression is increased in animals with increased sympathetic activity (Plochocka-Zulinska and Krukoff, 1997). These results indicate a potential role for NO in regulation of HPA axis activity and sympathetic output.

### **1.6.3. Effects of estrogen on production of NO**

Direct evidence of the effects of estrogen on circulating NO concentrations is provided by data that showed that ERT in postmenopausal woman increases circulating nitrate/nitrite levels (Lopez-Jaramillo et al.,



1996; Best et al., 1998). Further evidence showed that BSA conjugated  $17\beta$ -estradiol stimulated NO release from the median eminence, and tamoxifen (ER inhibitor) antagonized these effects. These results suggest a membrane surface receptor (Prevot, 1999). It was also found that this NO release is mediated by vascular endothelial cells (eNOS), and leads to GnRH release (Prevot, 1999). More studies have shown that the effect of estrogen on eNOS is mediated by  $ER\alpha$  via nongenomic means (Shaul, 2000).

A mechanism for the stimulation of eNOS by estrogen involves the estrogen receptor complex which contains heat shock protein 90 (hsp90; Segnitz and Gehring, 1995); eNOS activity is increased in a dose-dependent manner in the presence of hsp90 (Garcia-Cardena et al., 1998). Russell et al. (2000) showed that this increase in eNOS activity due to hsp90 is promoted by  $17\beta$ -estradiol. Further evidence is offered by the fact that human arterial endothelial cells contain cell-surface estrogen receptors that stimulate NO release upon estrogen treatment (Stefano et al., 2000), and that eNOS in the endothelium of blood vessels increases as estrogen levels peak during the menstrual cycle (Taguchi et al., 2000). These data suggest a stimulatory effect of estrogen upon eNOS protein and activity. Even though hypoxic stress increases eNOS expression in vasculature, estradiol treatment does not augment expression (Resta, et al., 2001). As of yet, little is known about the effects of estrogen on central NO during psychological (restraint) stress.

Estradiol attenuates plasma nitrate levels (NO via iNOS) upon the injection of LPS (Kauser et al., 1997). Similarly, in dissected aortae of OVX



rats, iNOS protein expression was increased, but estradiol treatment inhibited the induction of iNOS (Tamura et al., 2000). This data suggests that estrogen can inhibit iNOS peripherally. This is further supported by the evidence that estradiol reduced the expression of iNOS in vascular endothelium in uremic rats (Noris et al., 2000).

Conflicting data exists for the effects of estrogen treatment on nNOS. It has been shown that estrogen down-regulates nNOS in rat anterior pituitary cells (Qian et al., 1999), whereas estradiol treatment of both intact male and female rats increases nNOS activity (guinea pig heart, kidney, skeletal muscle, and cerebellum) and mRNA levels in female skeletal muscle (Weiner et al., 1994).

## 1.7. Hypotheses

The experiments in this thesis were designed to test the hypotheses: **1) Estrogen attenuates stress-induced (psychological) sympathetic output and HPA axis activity.** To determine the effects of restraint stress on sympathetic output ovariectomized female rats with estrogen replacement (OVX-E) or vehicle (OVX-V), intact females, and males were subjected to restraint stress. Throughout the thesis comparisons were made between OVX-V and OVX-E animals, OVX-E and intact females, and intact males and females to determine effects of estrogen treatment, ovariectomy and gender, respectively, on HPA axis and sympathetic responses to restraint stress. Blood





pressure and heart rate responses to stress were recorded, and sympathetic output was further assessed by measuring tyrosine hydroxylase (TH) mRNA levels in the locus coeruleus (LC). To measure the effects of stress on the HPA axis, adrenocorticotropin hormone (ACTH) plasma levels and corticotropin releasing hormone (CRH) mRNA in the paraventricular nucleus (PVN) of the hypothalamus were quantified in all groups of animals.

Since NO production is stimulated during stress to restore homeostasis to both sympathetic output and the HPA axis, a second hypothesis was tested: **2) Estrogen mediates attenuation of stress-induced sympathetic output and HPA axis activity via NO.** Experimental animal treatments similar to those used to test the first hypothesis were used. To determine the number of neurons containing nNOS, neurons were stained using NADPH diaphorase (NADPHd) histochemistry (Hope et al., 1991). To quantify NO production, a nitrate/nitrite assay (Salter et al., 1996) was used to determine NO concentration in the brainstem and the hypothalamus.

Since estrogen replacement in humans is usually for an extended time, preliminary studies were done to test a third and fourth hypotheses, **3) Long-term estrogen replacement therapy (8 weeks) attenuates sympathetic drive and HPA axis activity during restraint stress,** and **4) Long-term estrogen replacement therapy attenuates sympathetic drive and the HPA axis via NO.** OVX female rats with 8 week estrogen (LTE) or vehicle (LTV) replacement were subjected restraint stress, and blood pressure and heart rate responses and HPA axis activity (ACTH plasma levels) were recorded. To

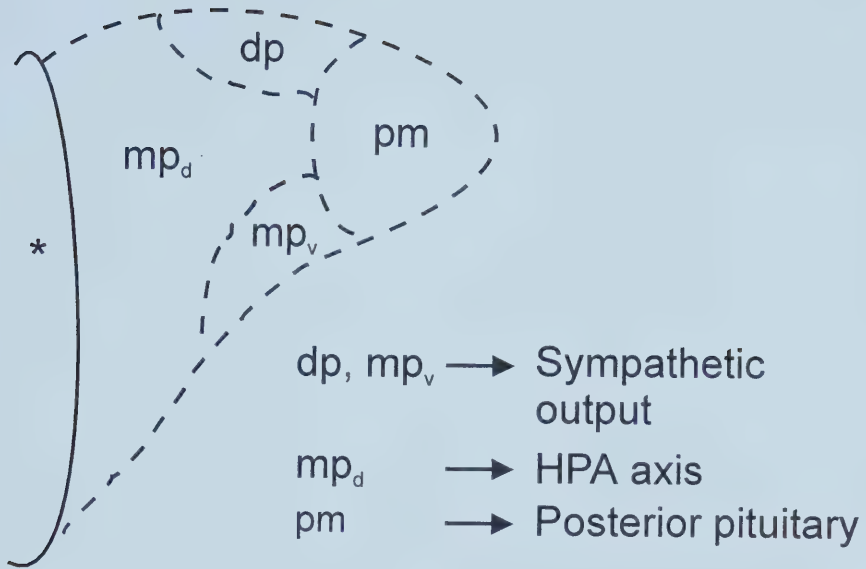


quantify NO production, a nitrate/nitrite assay was performed to determine NO concentration in the hypothalamus and brainstem, and NADPHd histochemical staining of PVN neurons was used to determine the number of neurons containing nNOS.



1000  
1000

Figure 1: Schematic diagram of the middle paraventricular nucleus (PVN) of the hypothalamus showing the dorsal parvocellular subdivision (dp), dorsal medial parvocellular subdivision (mpd), ventral parvocellular subdivision (mpv), and posterior magnocellular subdivision (pm). The dp and mpv subdivisions are important for sympathetic output, the mpd subdivision is important for HPA axis activity, and neurons of the pm subdivision project to the posterior pituitary.







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Figure 2: Depiction of the baroreceptor reflex. Inputs onto the paraventricular nucleus of the hypothalamus (PVH) from the locus coeruleus (LC), nucleus tractus solitarius (NTS), and ventrolateral medulla (VLM) are shown in the left panel. In turn, the PVH then projects onto the LC, NTS, VLM, and the intermediolateral cell column (IML). The IML then can affect the heart and blood vessels via sympathetic ganglia (Loewy, 1981).

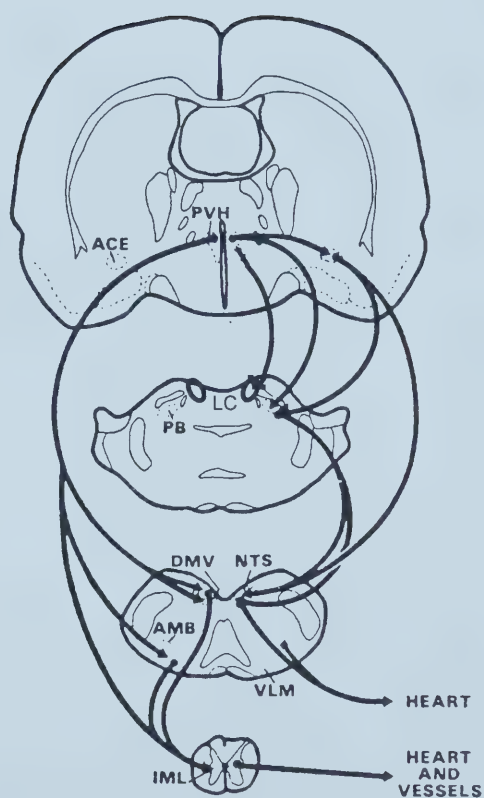
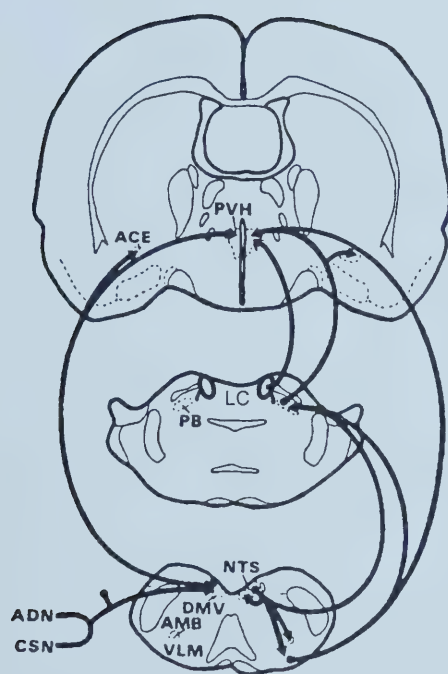
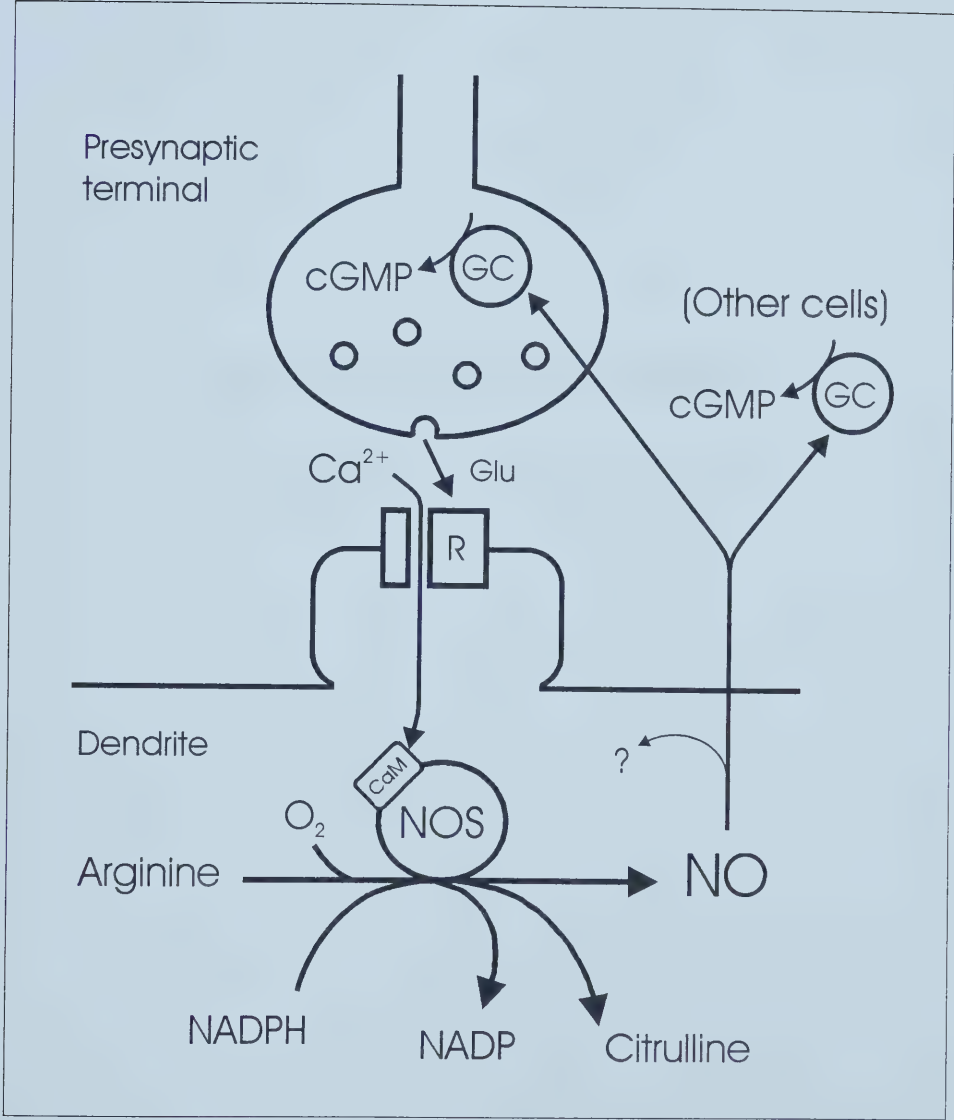






Figure 3: Schematic diagram of nitric oxide production. Glutamate (Glu) is released from the presynaptic terminal and acts upon NMDA receptors (R) in the postsynaptic element. Opening of the channel allows calcium ( $\text{Ca}^{2+}$ ) to enter and to activate nitric oxide synthase (NOS) through calmodulin (CaM) binding. NO is formed from arginine in the presence of oxygen ( $\text{O}_2$ ) and nicotinamide adenine dinucleotide phosphate (NADPH). NO then diffuses to the presynaptic terminal or neighboring cells and acts on guanylyl cyclase (GC) to stimulate production of cGMP (Krukoff, 1999).







## **CHAPTER 2**

### **MATERIALS AND METHODS**



## 2.1. Animals

Female and male Sprague-Dawley rats were purchased from the Biosciences Animal Center, University of Alberta. Rats were between 200-300 g, and had access to food and water *ad libitum*. The rats were housed in a room at 21°C with a 12 hr light: 12 hr dark lighting cycle. All procedures were done according to the ethical protocols of Health Sciences Lab Animal Services.

## 2.2 Instrumentation

Animals were anesthetized using intraperitoneal sodium pentobarbitol [Somnotol (M.T.C. Pharmaceuticals, Hamilton, ON)], 45 mg/kg (females) and 55 mg/kg (males) and methoxyflurane [Metofane (Janssen Pharmaceutica, Toronto, ON)], and received 15 µg buprenorphine hydrochloride (Buprenex Injectable, Reckitt and Colman Pharmaceutical Inc., Richmond, VA) and ampicillin (Sigma, St. Louis, MO) intramuscularly.

### 2.2.1 Arterial cannulation

Arterial cannulations were done as described previously (Yang and Krukoff, 2000). The abdominal cavity of each rat was opened and organs were moved aside to expose the vena cava and descending aorta. Polyethylene tubing of internal diameter 0.28 mm and external diameter 0.61 mm (PE 10; Becton Dickinson, Sparks, MD)



was inserted into the dorsal aorta and sutured to the dorsal musculature. The cannula was then exteriorized under the skin to an area between the scapulae where it was attached to an external polyethylene tube (PE 10) via a constructed pedestal with 27-gauge stainless steel tubing (Small Parts Inc. Miami Lake, FL). Polyvinylpyrrolidone (PVP-40) was used to partially fill the cannula after surgery to prevent bleeding due to arterial pressure, and the tube was capped with silastic tubing.

### *2.2.1 Ovariectomy and Estrogen Replacement*

During the same surgical session, ovaries were removed from female rats, and a pellet of  $17\beta$ -estradiol or placebo (Innovative Research of America, Sarasota, FL; 21 day release, 0.25 mg/pellet) was wrapped in surgical sponge and implanted subcutaneously between the scapulae. After experimentation and animal processing, the pellets were removed and placed into sterile petri dishes and were re-used up to 2 more times. Intact diestrus animals were processed during the diestrus phase of the estrus cycle. Male rats were left intact with no estrogen or placebo treatment. Long-term treated rats also received  $17\beta$ -estradiol or placebo subcutaneous pellets (Innovative Research of America, Sarasota, FL; 60 day release, 0.25 mg/pellet).



## 2.3 Experimental design

### 2.3.1 Restraint

Animals were allowed to recover for 4-5 days after surgery and pellet implantation. Rats were then restrained in a hemicylindrical ventilated plexiglass tube. Animals used for blood pressure/heart rate measurements, *in situ* hybridization, and neuronal staining underwent a “1 hour restraint, 1hour rest, 1 hour restraint, 1hour rest” paradigm (Yang and Krukoff, 2000). Animals used for the nitrate/nitrite assay underwent 1 hour of restraint only. Control measurements were taken from animals that were unrestrained.

### 2.3.2 Blood pressure (BP)/Heart Rate (HR) Measurements

Blood pressure and heart rate were measured by connecting the exteriorized arterial cannula to a pressure transducer. The transducer then relayed the cardiovascular information to a computer running WinDaq/Lite software (DATAQ Instruments, 1998) where the information was recorded. Baseline diastolic and systolic blood pressure measurements were recorded at -15, -10, -5, -1 and -0.25 minutes. Experimental diastolic and systolic BPs were also recorded at the time of restraint, 1, 3, 5 minutes afterwards, and every 5 minutes thereafter. During rest sessions, diastolic and systolic BP were recorded at the times of 0, 1, 3, 5 minutes,





and every 5 min thereafter. Mean arterial blood pressure (MAP, mmHg) was determined using the equation  $[(\text{systolic-diastolic})/3] + \text{diastolic}$ . Heart rate was determined directly using the WINDAQ software.

### *2.3.3 Nitrate/Nitrite Assay*

After 1 hour of restraint, animals were decapitated and the brain stem (cut approximately between -9.5 and -16 mm Bregma) and hypothalamus [cut approximately between 0 and -5 mm Bregma (rostral-caudal), 2 mm to either side of the midline (lateral), and 4 mm below dura (dorsal-ventral)] were collected. The tissue samples were then homogenized in 750  $\mu\text{l}$  phosphate buffered saline (PBS; 13.4 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  (Fisher Scientific, Nepean, ON), 8 g NaCl (BDH Inc., Toronto, ON), 1 L water; pH=7.4). Homogenates were centrifuged (11,000 g for 20 min), ultracentrifuged (53,000 g for 15 minutes) and ultrafiltered using 30 kDa molecular weight cut-off filters (Millipore MPS micropartition devices, centrifuged for 30 min at 2,000 g)(Yang and Krukoff, 2000).

Assays were done according to kit instructions (Cayman Chemical, Ann Arbor, MI) in duplicate for each sample. Enzyme co-factors (10  $\mu\text{l}$ ) and nitrate reductase (10  $\mu\text{l}$ ) were added to each sample, and incubated at room temperature. A colorimetric reaction occurred upon the addition of 50  $\mu\text{l}$  of each Griess Reagent R1 and Griess Reagent R2. The absorbance at 540 nm was read using an Elisa plate reader (MTX Lab System Inc., McLean, VA) as described previously (Yang and Krukoff, 2000).



## 2.4. Tissue Preparation

Anesthetized animals were perfused through the left ventricle of the heart with ice-cold 0.9% saline followed by ice-cold 4% paraformaldehyde. After perfusion, brains were removed and transferred into a solution of 2% paraformaldehyde and 10% sucrose for 1 hour. The brains were then transferred into 20% sucrose and stored overnight at 4 °C.

Brains were cut into 3 sets of 40 µm sections, one floating in PBS for histochemistry, and two thaw-mounted onto Superfrost/Plus slides (Fisher Scientific) for *in situ* hybridization. After air-drying, the thaw-mounted sections were frozen at -70 °C for future analysis.

## 2.5 *In situ* hybridization

Protocols for cRNA probe synthesis, *in situ* hybridization, and autoradiography have been described previously (Krukoff et al., 1999; Yang and Krukoff, 2000). Slides were removed from the -70°C freezer, and air-dried for 1 hour. They were then washed in 4% paraformaldehyde, 1 x PBS, 1 x PBS, Proteinase K buffer [20 µg/ml proteinase K (Promega), 10 ml 1 M Tris /HCl (pH 8.0), 4 ml 0.5 M EDTA, and 186 ml water], 1 x PBS, 4% paraformaldehyde, TEA/acetic anhydride solution [2.6 ml 0.1 M TE, 600 µl acetic anhydride (BDH Inc.), 2.4 ml 5 N NaOH (Fisher Scientific), and 250 ml water], 30ml 3 M sodium acetate trihydrate (NaOAC; BDH Inc.) in 170ml 70% ethanol, 30 ml 3 M NaOAC in 170 ml 80% ethanol, 95%



ethanol and 100% ethanol. The slides were then air-dried for 2 hours, and  $^{35}\text{S}$ -UTP labeled cRNA probe ( $3 \times 10^6$  cpm/slide) was added to each slide. The slides were coverslipped and incubated at  $46^\circ\text{C}$  overnight. The slides were uncovered and put into 2 x standard saline citrate (SSC) for 20 minutes, RNase inhibitor in STE buffer at  $37^\circ\text{C}$  for 30 minutes, 2 x SSC at  $45^\circ\text{C}$  for 40 minutes, and a final wash in 0.1 x SSC at  $65^\circ\text{C}$  for 50 minutes to reduce non-specific binding of the probe.

The slides were allowed to air dry, and exposed to X-ray film (X-OMAT AR, Kodak) for 2-3 days. The slides were then dipped in NTB-2 Kodak photographic emulsion (diluted 1:1 with water) and stored in the dark at  $4^\circ\text{C}$  for 14-21 days for autoradiography. The slides were placed into D-19 Kodak developer for 5 minutes followed by a brief wash in water and 3 minutes in Kodak fixer with hardener. After washing the slides under running water for 40 minutes, tissue sections were stained with cresyl violet, and dehydrated using increasing concentrations of ethanol and xylene. The sections were then coverslipped for image analysis. Sections were analyzed with light and darkfield microscopy to quantify silver grain deposition.

### *2.5.1 cRNA Probes*

Antisense corticotropin releasing hormone (CRF) cRNA probes contained the +1283 to +2048 region of the peptide in the p-GEM-3Z plasmid, and were generated by linearizing with Hind III (Promega), and transcribed with T7 RNA polymerase (Promega). CRF sense probes were synthesized by linearizing with SacI (Promega), and transcribing with SP6 RNA polymerase (Promega). Antisense tyrosine



hydroxylase (TH) cRNA probes contained the +14 to +1165 region of the enzyme (Grima et al., 1985) in T-GEM-7Zf plasmid (2.5 µg/µl) linearized with ApaI (Promega), and transcribed with SP6 RNA polymerase (Promega). TH sense probes were made by linearizing with Hind III, and transcribed with T7 RNA polymerase (Promega).

## 2.6 Histochemistry

### 2.6.1. NADPH diaphorase histochemistry

Procedures for NADPH diaphorase histochemistry have been previously described (Yang et al., 1999). Floating sections were washed in PBS, and transferred to a solution of 20 ml 0.6 % Triton X-100/PBS with 1 mg/ml nicotinamide adenine dinucleotide phosphate, reduced form (NADPH; Sigma), mixed with 0.3 mg/ml nitroblue tetrazolium (Sigma) dissolved in 500 µl dimethylsulfoxide (DMSO; Caledon). Sections were incubated at 42°C for 25 minutes, transferred into PBS and mounted onto slides. After air drying, slides were coverslipped using Elvanol (Mowiol, Calbiochem; 10 g dissolved in 40 ml PBS and 20 ml glycerol (BDH Inc., Toronto, ON)). Analysis was done using a Zeiss light/fluorescence microscope.





## 2.7 Radioimmunoassay (RIA)

### 2.7.1 *Adrenocorticotropin hormone (ACTH)*

Blood samples were collected from the vena cava immediately before perfusion, put into tubes containing EDTA, and centrifuged at 6000 rpm for 6 min. The plasma was decanted and frozen at  $-70^{\circ}\text{C}$ . Radioimmunoassay (RIA) was done according to instructions provided in the ACTH RIA kit (ICN Biomedical, Inc., Costa Mesa, CA). Briefly, to 100  $\mu\text{l}$  of duplicate samples, ACTH anti-serum and hACTH- $\text{I}^{125}$  were added and incubated at  $4^{\circ}\text{C}$  overnight. The following day, 500  $\mu\text{l}$  precipitant solution was added to each tube. Tubes were then centrifuged at 1000 g for 15 minutes. The supernatant was decanted using a vacuum, and the radioactivity of the precipitant from each sample was quantified using a gamma counter.

### 2.7.2 *Estrogen*

Blood samples were collected from the vena cava immediately before perfusion, put into tubes containing EDTA, and centrifuged at 6000 rpm for 6 min. The plasma was decanted and frozen at  $-70^{\circ}\text{C}$ . Estradiol RIA was performed according to kit instructions (ICN Biomedicals, Inc., Costa Mesa, CA). To 50  $\mu\text{l}$  of duplicate samples, estradiol- $\text{I}^{125}$  and anti-estradiol was added. Tubes were incubated at  $37^{\circ}\text{C}$  for 90 minutes, then precipitant solution was added to each tube, and



centrifuged at 1000 g for 20 minutes at 4°C. The supernatant was decanted and the radioactivity of the precipitant was counted in a gamma counter.

## **2.8 Analysis**

The numbers of NADPHd-positively stained neurons in the PVN were counted, separating the rostral (between -1.70 and -1.55 mm Bregma), middle (between -1.85 and -1.70 mm Bregma) and caudal (between -2.00 and -1.85 mm Bregma) subdivisions (Krukoff et al., 1995). Subdivisions of middle and caudal sections were chosen according to areas previously defined (Yang and Krukoff, 2000). Stained neurons were counted when a nucleus was discernible. Data were expressed as number of positively stained neurons per section where an average of 1-3 sections was used per animal.

Quantification of mRNA levels in the middle PVN (CRF mRNA) and LC (TH mRNA) was done under darkfield microscopy using a digitizing scanning camera (DC330, DAGE MTI Inc., Michigan, MI) coupled to a computer running imaging software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). The software determined the percentage of area covered by silver grains (no units). The area used for quantifying positive signal was kept the same for each region, and for the background measurements of an area ventrolateral to the PVN and/or LC. An average of 1-3 sections was used per animal (Yang and Krukoff, 2000).



### *2.8.1 Statistics*

Graphs were produced in Excel 2000 (Microsoft, 2000). First, MAP and HR data were calculated as a percentage of baseline for individual animals. Data were then expressed as means  $\pm$  standard error of those means. Comparisons between multiple groups were done using a one-way analysis of variance (ANOVA) test followed by a Neuman-Keuls post-hoc test. Comparisons between an experimental treatment and controls were done using a Student's t-test. Statistics were performed using GraphPad Prism (GraphPad Software, San Diego, CA).  $P < 0.05$  indicated statistical significance.



**CHAPTER 3**

**RESULTS**





OVX-E rats had plasma estrogen concentrations of  $66.2 \pm 14.2$  pg/ml, while OVX-V rats had plasma estrogen concentrations of  $18.7 \pm 1.8$  pg/ml. Intact diestrus rats had plasma estrogen concentrations of  $34.89 \pm 14.04$  pg/ml. LTE rats had plasma estrogen concentration of  $28.15 \pm 4.3$  pg/ml, while LTV rats had plasma estrogen concentrations of  $22.09 \pm 1.1$  pg/ml.

### **3.1. Cardiovascular responses of OVX-V, OVX-E, diestrus and male rats to 4-hr restraint stress protocol**

#### *3.1.1. Controls: Blood pressure and heart rate in unstressed OVX-V, OVX-E, diestrus and male rats*

MAP and HR of conscious unrestrained rats did not change within each group, and were not significantly different among groups (Figure 4). However, there is a trend towards higher HR in male unrestrained control animals during the last two hours compared to the other groups (OVX-V, OVX-E, and diestrus) (Figure 4B).

#### *3.1.2. Effects of restraint on blood pressure and heart rate*

Upon restraint stress, MAP of OVX-V rats significantly increased compared to unrestrained controls ( $161.3 \pm 6.4$  % vs.  $95.2 \pm 3.5$  %), dropped approximately 40% from the peak in less than 1 minute, but remained elevated for 20 minutes during the first hour of restraint. When the rats were removed from the restraint tube,



again MAP increased significantly ( $146.4 \pm 7.5$  % vs.  $96.6 \pm 4.2$  %), dropped approximately 35% from the peak in less than 1 minute, and remained elevated for 5 minutes. When the rats were put into the restraint tube again, MAP rose significantly ( $146.9 \pm 3.6$  % vs.  $95.6 \pm 9.2$  %), dropped approximately 35% from the peak in less than 1 minute, and remained elevated for 10 minutes. When the rats were removed from the restraint tube for the second time, MAP rose significantly ( $141.6 \pm 5.0$  % vs.  $99.9 \pm 7.3$  %), dropped approximately 25% from the peak in less than 1 minute, and remained elevated for 5 minutes (Figure 5A). Similarly, HR of OVX-V rats rose significantly above controls upon restraint ( $117.1 \pm 2.8$  % vs.  $95.9 \pm 1.9$  %) and began to decrease, but remained elevated for 20 minutes. Upon removal from the restraint tube, HR rose significantly ( $119.1 \pm 5.1$  % vs.  $97.6 \pm 0.01$  %), began to decrease, and remained high for 20 minutes. When the rats were put back into the restraint tube, HR rose significantly ( $120.3 \pm 5.2$  % vs.  $86.4 \pm 3.6$  %), began to decrease, and remained elevated for 5 minutes. Upon removal from the tube, rats showed a significant rise in HR ( $119.8 \pm 4.9$  % vs.  $89.1 \pm 8.1$  %) that dropped to baseline after 5 minutes (Figure 6A).

Following the same pattern as the MAP and HR of OVX-V rats, the MAPs of restrained OVX-E rats significantly increased above unrestrained controls upon initial restraint ( $154.2 \pm 2.5$  % vs.  $99.0 \pm 7.2$  %) and then subsided after 3 minutes. Upon removal from the tube, MAP rose significantly for less than 1 minute ( $153.9 \pm 9.3$  % vs.  $98.9 \pm 4.0$  %). When the rats were put into the restraint tube again, MAP rose significantly ( $153.7 \pm 6.0$  % vs.  $91.9 \pm 5.2$  %), began to decrease from the peak pressure, and remained elevated for 3 minutes. Upon removal from the restraint tube,



MAP rose significantly ( $156.5 \pm 6.7 \%$  vs.  $93.8 \pm 4.1\%$ ), began to decrease, and remained elevated for 10 minutes (Figure 5B). The HR of OVX-E rats also increased significantly above unrestrained controls upon restraint ( $116.6 \pm 5.6 \%$  vs.  $99.4 \pm 4.0 \%$ ) and remained elevated for 15 minutes. When the rats were removed from the restraint tube, HR rose significantly ( $119.0 \pm 3.5 \%$  vs.  $92.3 \pm 3.6 \%$ ) and remained elevated for 1 minute. When the animals were put back into the tube, HR rose, but did not reach a significant difference. When removed from the restraint tube again, HR rose significantly ( $116.3 \pm 7.1 \%$  vs.  $92.7 \pm 5.6 \%$ ) and remained higher for 1 minute (Figure 6B).

Upon restraint, diestrus rats showed significantly higher MAPs ( $154.2 \pm 2.5 \%$  vs.  $99.3 \pm 3.8 \%$ ) that fell to unrestrained control levels in 1 minute. When the rats were removed from the restraint tube, MAP rose significantly ( $153.9 \pm 9.3 \%$  vs.  $104.1 \pm 5.9 \%$ ), and in less than 1 minute, fell back to baseline. After 15 minutes of rest, the rats exhibited a significantly lower MAP than control animals for 35 minutes. When the animals were placed back into the restraint tube, MAP rose significantly ( $153.7 \pm 6.0 \%$  vs.  $100.4 \pm 6.3 \%$ ) and in less than 1 minute fell back to control values. After 15 minutes of restraint, the rats exhibited significantly lower MAP than control animals for 20 minutes. When the animals were removed from the restraint tube for the second time, MAP rose significantly ( $156.5 \pm 6.7 \%$  vs.  $97.5 \pm 3.6 \%$ ) and in less than 1 minute fell back to control levels. After 15 minutes of rest, the rats exhibited a significantly lower MAP than control animals for 10 minutes (Figure 5C). The HR for diestrus animals rose significantly from unrestrained controls upon restraint ( $116.6 \pm 5.6 \%$  vs.  $100.3 \pm 3.4 \%$ ) for less than 1 minute.



When the animals were removed from the restraint tube, there was no significant increase in HR, although there was a trend showing a slight increase. When the animals were put into the restraint tube for a second time, HR did not increase significantly. Throughout this hour of restraint there were no significant differences compared to controls, but there was a trend of steadily increasing HR. When the animals were removed from the restraint tube, HR was significantly higher than in control animals ( $116.3 \pm 7.1$  % vs.  $91.5 \pm 6.1$  %) throughout the hour of rest. Again, HR showed a trend of a steady increase (Figure 6C).

The male rats exhibited significantly increased MAP upon restraint when compared to unrestrained controls ( $145.8 \pm 5.2$  % vs.  $96.4 \pm 2.3$  %) for 10 minutes. When removed from the restraint tube, MAP was significantly elevated ( $158.8 \pm 8.4$  % vs.  $98.3 \pm 2.7$  %) for 5 minutes. When the animals were put back into the restraint tube, MAPs rose significantly ( $145.4 \pm 6.3$  % vs.  $99.7 \pm 6.2$  %) and remained elevated for 1 minute. When the rats were removed from the tube for the second time, MAPs rose significantly ( $161.7 \pm 7.7$  % vs.  $105.7 \pm 7.1$  %), but immediately returned to control levels (Figure 5D). The HRs of male rats also rose significantly from unrestrained controls upon restraint ( $125.3 \pm 3.3$  % vs.  $96.4 \pm 1.7$  %) and remained elevated for 20 minutes. When the rats were taken out of the restraint tube, HR rose significantly ( $117.3 \pm 3.2$  % vs.  $94.6 \pm 2.4$  %) for 10 minutes. When the rats were put back into the restraint tube, there was no significant difference in HR, although there appeared to be a slight increase. When the animals were removed from the restraint tube, HR rose significantly ( $126.1 \pm 2.6$  % vs.  $103.9 \pm 4.4$  %) and remained elevated for 3 minutes (Figure 6D).





### 3.1.3. Comparisons of MAP and HR among restrained rats

The MAP and HR of all four restrained animal groups (OVX-V, OVX-E, diestrus, and male) appear in Figure 7A and 7B respectively. No significant differences were found between MAPs of OVX-V rats and OVX-E rats (Figure 8A) or between HRs for the two groups (Figure 9A). When comparing restrained diestrus rats and OVX-E rats, it is seen that diestrus females had significantly lower MAPs than OVX-E rats ( $87.3 \pm 4.7 \%$  vs.  $107.0 \pm 5.6 \%$ ) beginning 3 minutes after removal from the restraint tube (Figure 8B). When comparing HRs of these two animal groups, there are few significant differences (Figure 9B). Males had significantly higher MAPs than diestrus females ( $113.9 \pm 3.6 \%$  vs.  $87.3 \pm 4.7 \%$ ) beginning 3 minutes after removal from the restraint tube (Figure 8C). The HR comparison shows a trend towards a higher male HR than diestrus HR (Figure 9C). Comparing restrained male rats to OVX-V rats shows no significant differences in either MAP (Figure 8D) or HR (Figure 9D), although starting 15 minutes after the second removal from the restraint tube, there is a trend for higher HR in males.

When comparing the MAPs of restrained LTV rats to LTE rats, LTV rats show a trend towards higher MAP than LTE rats with significantly higher MAP at a few points during restraint periods (Figure 10A). LTV rats showed significantly higher HR than LTE rats (Figure 10B).



### 3.2. Effects of Restraint on mRNA Levels of Tyrosine Hydroxylase in the LC

The comparison among all 4 unrestrained controls showed significantly lower TH mRNA in the LC of OVX-V ( $0.05 \pm 0.02$ ) and male ( $0.13 \pm 0.04$ ) rats when compared to diestrus animals ( $0.37 \pm 0.09$ ) (Figure 11A). When comparing all 4 stressed animal groups, it is seen that there are no significant differences (Figure 11B). When comparing stressed animal groups to the corresponding unrestrained control animals, a significant increase in TH mRNA was seen in the LC in OVX-V ( $0.27 \pm 0.03$  vs.  $0.05 \pm 0.02$ ) and male animals ( $0.41 \pm 0.03$  vs.  $0.13 \pm 0.04$ ) upon the 4-hr restraint stress protocol (Figure 12).

Figure 13 shows the TH mRNA signal in the LC of both unrestrained (Figure 13A) and restrained (Figure 13B) male rats. Positive signal is located throughout the neurons of the LC. Figure 13B shows significantly higher signal than Figure 13A.

### 3.3. Effects of Restraint on Nitric Oxide Production in the Hypothalamus and Brain Stem

In the hypothalamus of unrestrained diestrus rats, a significantly lower nitrate/nitrite concentration was observed ( $0.58 \pm 0.14 \mu\text{M}$ ) compared to OVX-V ( $1.46 \pm 0.56 \mu\text{M}$ ), OVX-E ( $1.66 \pm 0.55 \mu\text{M}$ ) and male rats ( $2.87 \pm 0.35 \mu\text{M}$ ) (Figure 14A). There were no significant differences in nitrate/nitrite concentration among all 4 groups of control animals in the brain stem (OVX-V, OVX-E, diestrus, male) (Figure 14A). After 1 hour of stress, there were no significant differences among any



of the experimental groups in the hypothalamus (OVX-V, OVX-E, diestrus, male). In the brain stem of the 4 groups, OVX-E rats had a significantly higher nitrate/nitrite concentration ( $5.52 \pm 1.0 \mu\text{M}$ ) than either OVX-V ( $2.1 \pm 0.14 \mu\text{M}$ ) or diestrus animals ( $2.69 \pm 0.33 \mu\text{M}$ ) after 1 hour of stress (Figure 14B).

In both the hypothalamus and brain stem of OVX-V rats, no significant differences in nitrate/nitrite concentrations are seen between controls and stressed animals (Figure 15A). In contrast, OVX-E rats show a significant increase in nitrate/nitrite concentration in both the hypothalamus ( $1.66 \pm 0.55 \mu\text{M}$  vs.  $4.6 \pm 1.53 \mu\text{M}$ ) and the brain stem ( $2.32 \pm 0.4 \mu\text{M}$  vs.  $5.52 \pm 1.0 \mu\text{M}$ ) (Figure 15B). In the hypothalamus of diestrus females, there was a significant increase in nitrate/nitrite concentration ( $0.58 \pm 0.14 \mu\text{M}$  vs.  $2.05 \pm 0.39 \mu\text{M}$ ), but not in the brain stem (Figure 15C). No significant differences between controls and stressed males were seen in either the hypothalamus or the brain stem (Figure 15D). There is a significant decrease in nitrate/nitrite concentration in the hypothalamus of LTE animals compared to LTV animals ( $2.59 \pm 0.36 \mu\text{M}$  vs.  $3.94 \pm 0.26 \mu\text{M}$ ), whereas LTE rats had a significantly higher nitrate/nitrite concentration in the brain stem in comparison to LTV ( $2.59 \pm 0.36 \mu\text{M}$  vs.  $4.40 \pm 0.4 \mu\text{M}$ ) (Figure 16).

### **3.4. Effects of restraint on numbers of NADPHd positive neurons in the PVN**

In the magnocellular subdivision of the middle PVN among unrestrained rats, OVX-E control rats had significantly fewer NADPHd positive neurons than all the



other control groups. In the mpv division of the middle PVN, OVX-E and diestrus control rats had significantly lower numbers than male controls (Figure 17A).

The dp subdivision of the PVN in restrained OVX-E rats showed significantly more NADPHd stained neurons than all the other restrained groups. In both the mpv and mpd subdivisions of the middle PVN, both OVX-V and OVX-E restrained animals had significantly higher numbers than male rats. Restrained OVX-V and OVX-E rats had significantly higher numbers than diestrus animals in the mpd subdivision of the middle PVN (Figure 17B).

The PVN of OVX-V of stressed animals showed significantly higher numbers of NADPHd positive neurons than unrestrained controls in the dp, mpv, and mpd subdivisions of the middle PVN and the mp subdivision of the caudal PVN. No significant differences were seen in the rostral PVN or in the magnocellular neurons (Figure 18A). NADPHd staining of the PVN in OVX-E animals showed a significant increase in positively stained neurons in the rostral PVN, in all the subdivisions of the middle PVN, and in the lp subdivision of the caudal PVN. There was no significant difference in the mp subdivision of the caudal PVN (Figure 18B). Significantly increased numbers of NADPHd stained neurons were seen in stressed male animals in comparison to controls in the rostral PVN, and the magnocellular neurons of the middle PVN (Figure 18C). Significantly higher numbers of NADPHd stained neurons were seen in stressed diestrus animals in comparison to controls in the rostral PVN, both subdivisions of the caudal PVN, and the mpv subdivision of the middle PVN (Figure 18D). Figure 19 shows that LTE females have significantly more NADPHd stained neurons in the magnocellular neurons of the middle PVN,





when compared to LTV animals. There were no other significant differences in the PVN.

Figure 20 shows NADPHd staining of the middle PVN in OVX-E rats. Figures 20A and 20C are photographs of unrestrained OVX-E animals at low and high magnification, respectively. Figures 20B and 20D are photographs of restrained OVX-E animals at low and high magnification, respectively. Small numbers of NADPHd-positive neurons are found in all subdivisions of the middle PVN in unrestrained OVX-E animals (Figure 20A). Significantly more NADPHd-positive neurons are found in all of the subdivisions of the middle PVN in restrained OVX-E rats (Figure 20B).

### **3.5. Effects of Restraint on mRNA Levels of Corticotropin Releasing Factor in the PVN**

The comparison among all 4 unrestrained control groups showed significantly higher CRF mRNA levels in the dp and mpd subdivisions of the PVN in OVX-V and diestrus rats in comparison to male rats (Figure 21A). There is also a trend that all the unrestrained female groups show higher CRF mRNA levels than unrestrained males in dp, mpv and mpd subdivisions. When comparing all 4 stressed animal groups, there are no significant differences in CRF mRNA levels although there is a trend that OVX-V rats had higher CRF mRNA levels in the dp, mpv, and mpd than the other 3 groups (Figure 21B). Restrained diestrus animals had a trend of lower CRF mRNA signal throughout the PVN subdivision when compared to unrestrained



controls, and this decrease was significant in the mpd (Figure 22C). Restrained male animals showed a trend of increased CRF mRNA signal in all of the PVN subdivisions when compared with unrestrained controls, with a significant increase in the mpv (Figure 22D).

CRF mRNA signal in the middle PVN is shown in unrestrained (Figure 23A) and restrained (Figure 23B) diestrus female rats, and signal is also shown in unrestrained (Figure 23C) and restrained (Figure 23D) male rats. Positive signal is found within the dp, mpv, and mpd subdivisions all the groups. Significantly more signal is found in the unrestrained diestrus dp and mpd subdivisions compared to the unrestrained males (Figures 23A and 23C). Significantly less signal is found in the mpd subdivision of restrained diestrus rats when compared to unrestrained controls (Figures 23A and 23B). Significantly more signal is found in the mpv subdivision of restrained male rats when compared to unrestrained controls (Figures 23C and 23D).

### **3.6. Effects of Restraint on Plasma Adrenocorticotropin Hormone Levels**

No significant differences in plasma ACTH concentration were found among all 4 unrestrained control groups (Figure 24A). When comparing all 4 stressed animal groups, it is seen that the stressed males have a significantly lower plasma ACTH concentration ( $96.6 \pm 15.7$  pg/ml) than OVX-V ( $281.8 \pm 24.7$  pg/ml), OVX-E ( $228.8 \pm 32.9$  pg/ml) and diestrus animals ( $295.7 \pm 34.0$  pg/ml) (Figure 24B). When comparing control animals to the corresponding stressed animal groups, it was seen that only the OVX-V animals exhibited a significant increase in plasma ACTH



concentration ( $185.9 \pm 27.9$  pg/ml vs.  $281.8 \pm 24.7$  pg/ml) upon the 4-hr restraint stress protocol (Figure 25). There were no significant differences in plasma ACTH concentration between LTV and LTE rats (Figure 26).

### **3.7. Weight Gain exhibited by OVX-V, OVX-E, Intact Female, and Intact Male Rats Over 7 Weeks**

Weight gain was significantly different among groups. Males gained the most weight ( $79.1 \pm 6.2$  %), followed by OVX-V rats ( $67.0 \pm 9.6$  %), then intact females ( $48.8 \pm 1.2$  %), and OVX-E rats ( $21.4 \pm 5.3$  %) showed the least weight gain (Figure 27).







Figure 4: Measurements of MAP and HR in unrestrained control animal groups. A. Recordings of mean arterial pressure (MAP) in unrestrained OVX-V (filled boxes), OVX-E (open circles), diestrus (open boxes) and male (filled circles) rats. B. Recordings of heart rate (HR) in unrestrained OVX-V (filled boxes), OVX-E (open circles), diestrus (white boxes) and male (black boxes) rats. Data are calculated as a percentage of baseline, and are expressed as mean  $\pm$  SEM. \*, +, @, #, % indicate statistical significance between OVX-E and males, OVX-V and males, OVX-E and diestrus, OVX-V and diestrus, and diestrus and males respectively,  $p < 0.05$  (ANOVA).  $n = 4$  (males),  $n = 6$  (OVX-V),  $n = 4$  (OVX-E),  $n = 4$  (diestrus).

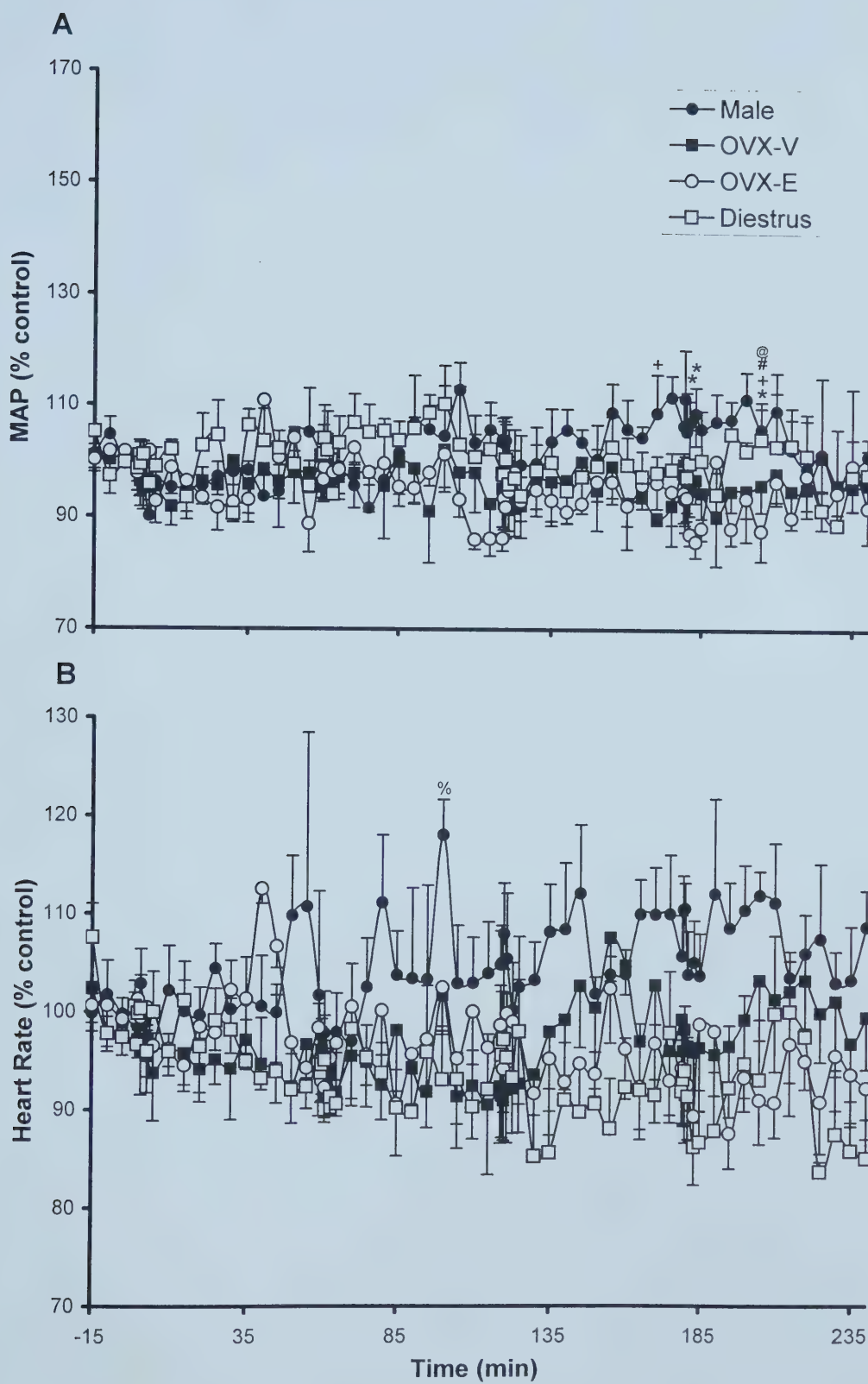






Figure 5: Measurements of MAP comparing restrained animals to unrestrained controls. Recordings of mean arterial pressure (MAP) in OVX-V (A), OVX-E (B), diestrus (C) and male (D) rats during restraint stress (filled circles) and in unrestrained controls (open boxes). Data are calculated as a percentage of baseline, and expressed as mean  $\pm$  SEM. Black arrows indicate the time at which rats were put into the restraint tube, white arrows indicate the time at which rats were taken out of the restraint tube. \* denotes significance,  $p < 0.05$  (Student's t-test). Unrestrained MAP:  $n = 4$  (male),  $n = 6$  (OVX-V),  $n = 4$  (OVX-E),  $n = 4$  (diestrus). Restrained MAP:  $n = 8$  (male),  $n = 7$  (OVX-V),  $n = 7$  (OVX-E),  $n = 5$  (diestrus).

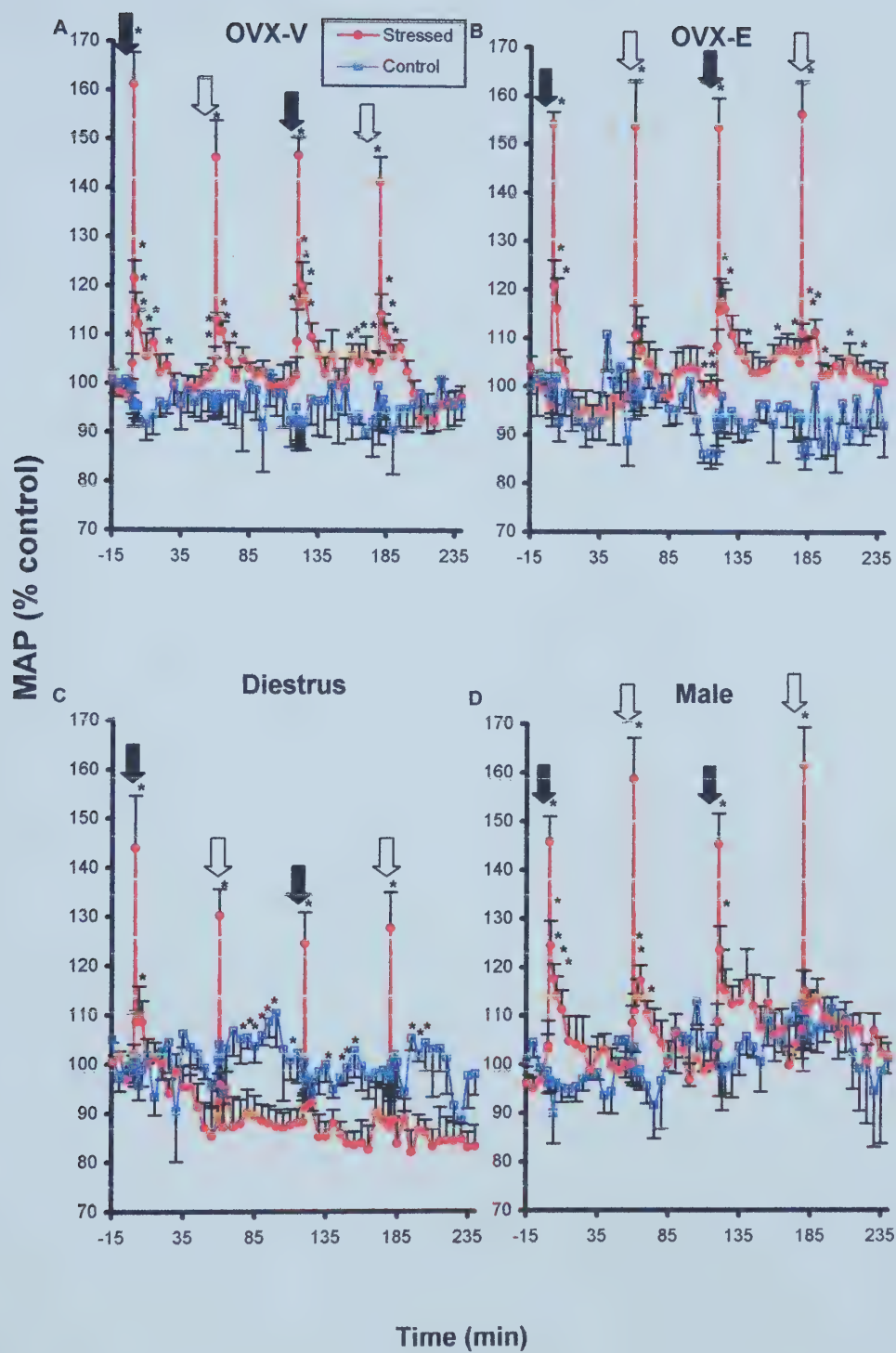








Figure 6: Measurements of HR comparing restrained animals to unrestrained controls. Recordings of heart rate (HR) in OVX-V (A), OVX-E (B), diestrus (C), and male (D) rats during restraint stress (filled circles) and in unrestrained controls (open boxes). Data are calculated as a percentage of baseline, and are expressed as mean  $\pm$  SEM. Black arrows indicate the time at which rats were put into the restraint tube, white arrows indicate the time at which rats were taken out of the restraint tube. \* denotes significance,  $p < 0.05$  (Student's t-test). Unrestrained HR:  $n = 4$  (male),  $n = 6$  (OVX-V),  $n = 4$  (OVX-E),  $n = 4$  (diestrus). Restrained HR:  $n = 8$  (male),  $n = 7$  (OVX-V),  $n = 7$  (OVX-E),  $n = 5$  (diestrus).

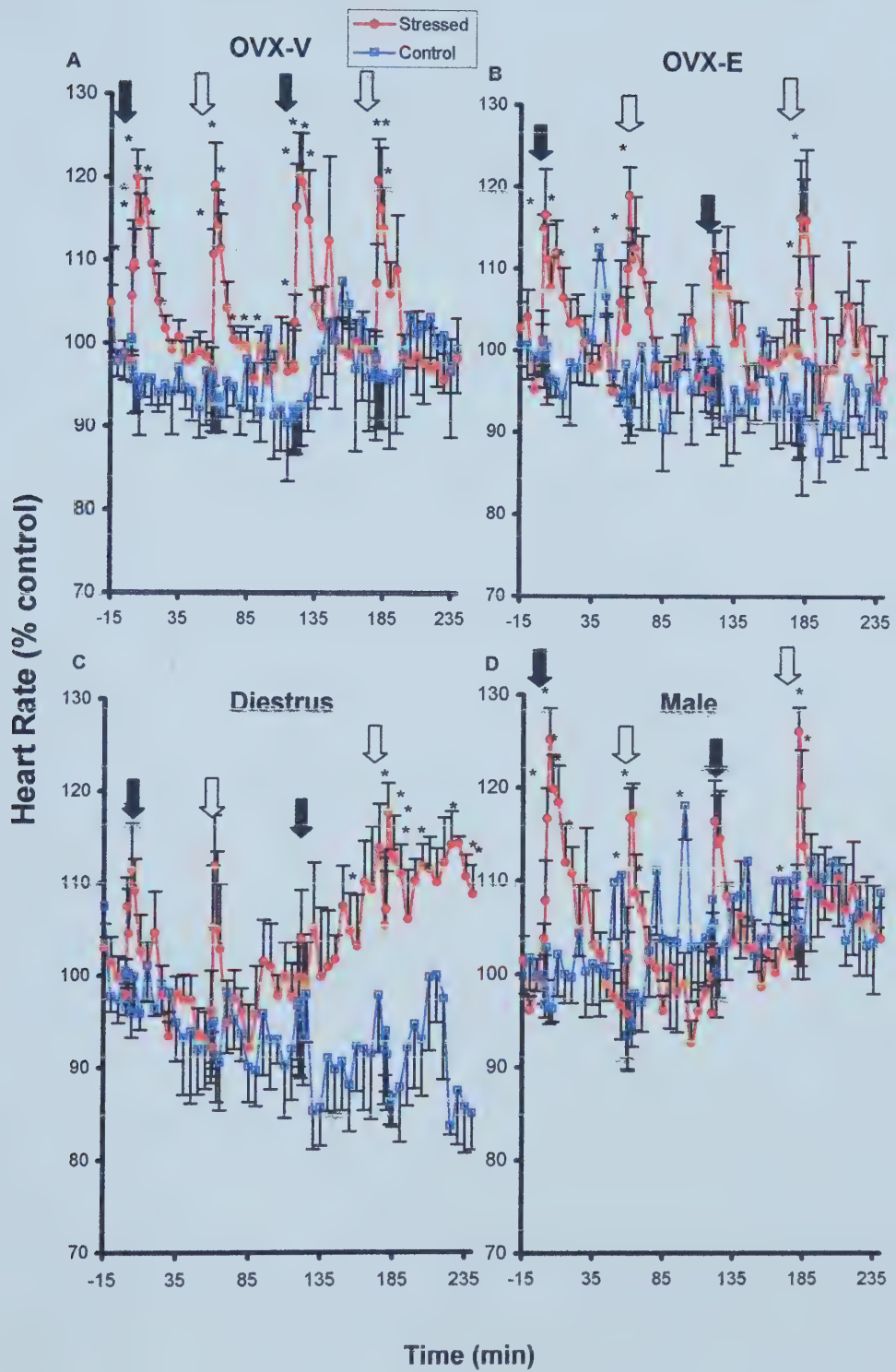






Figure 7: Measurements of MAP and HR in restrained animal groups. A. Recordings of mean arterial pressure (MAP) in restrained OVX-V (filled boxes), OVX-E (open circles), diestrus (open boxes) and male (filled circles) rats. B. Recordings of heart rate (HR) in restrained OVX-V (filled boxes), OVX-E (open circles), diestrus (open boxes) and male (filled circles) rats. Measurements are related to baseline, and data are expressed as mean  $\pm$  SEM. Black arrows indicate time at which rats were put into the restraint tube, white arrows indicate time at which rats were taken out of the restraint tube. n = 8 (male), n = 7 (OVX-V), n = 7 (OVX-E), n = 5 (diestrus).

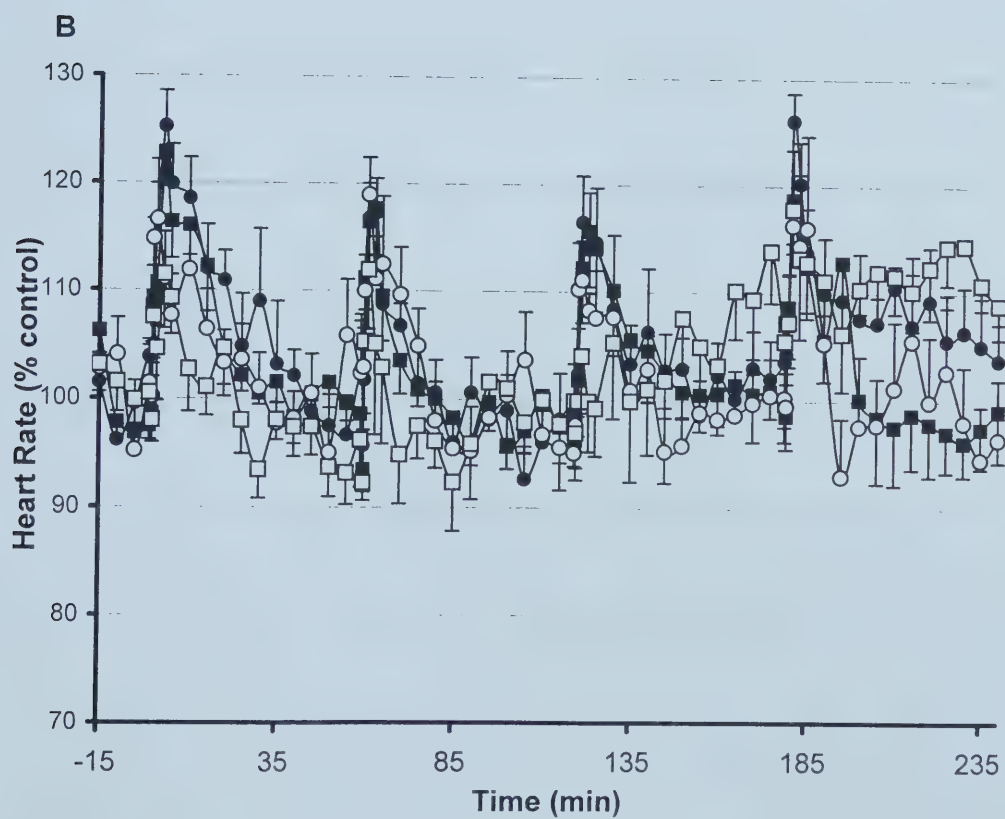
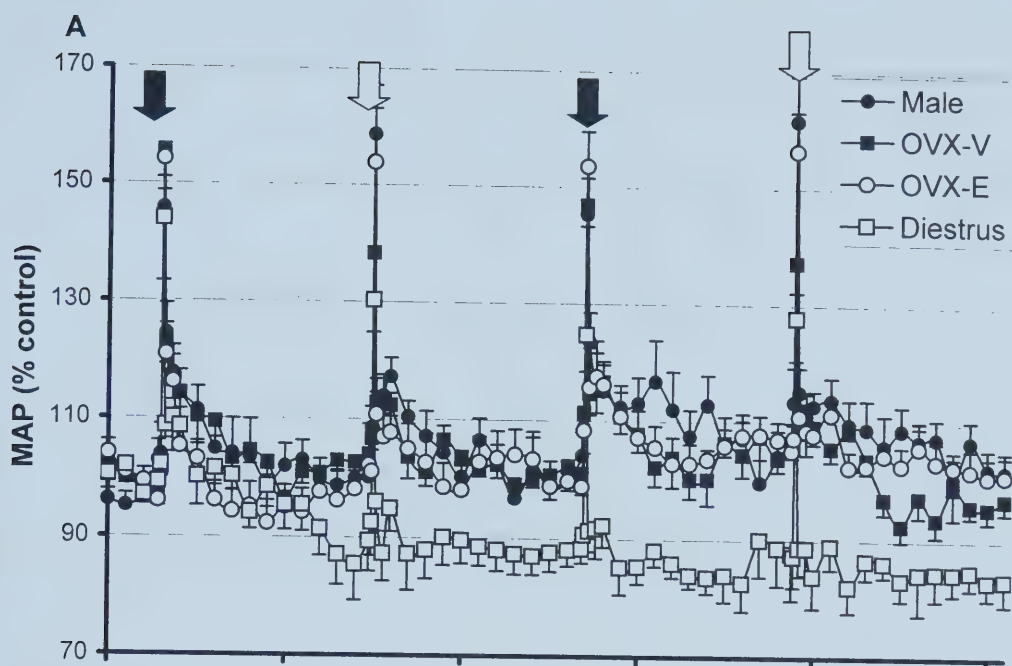
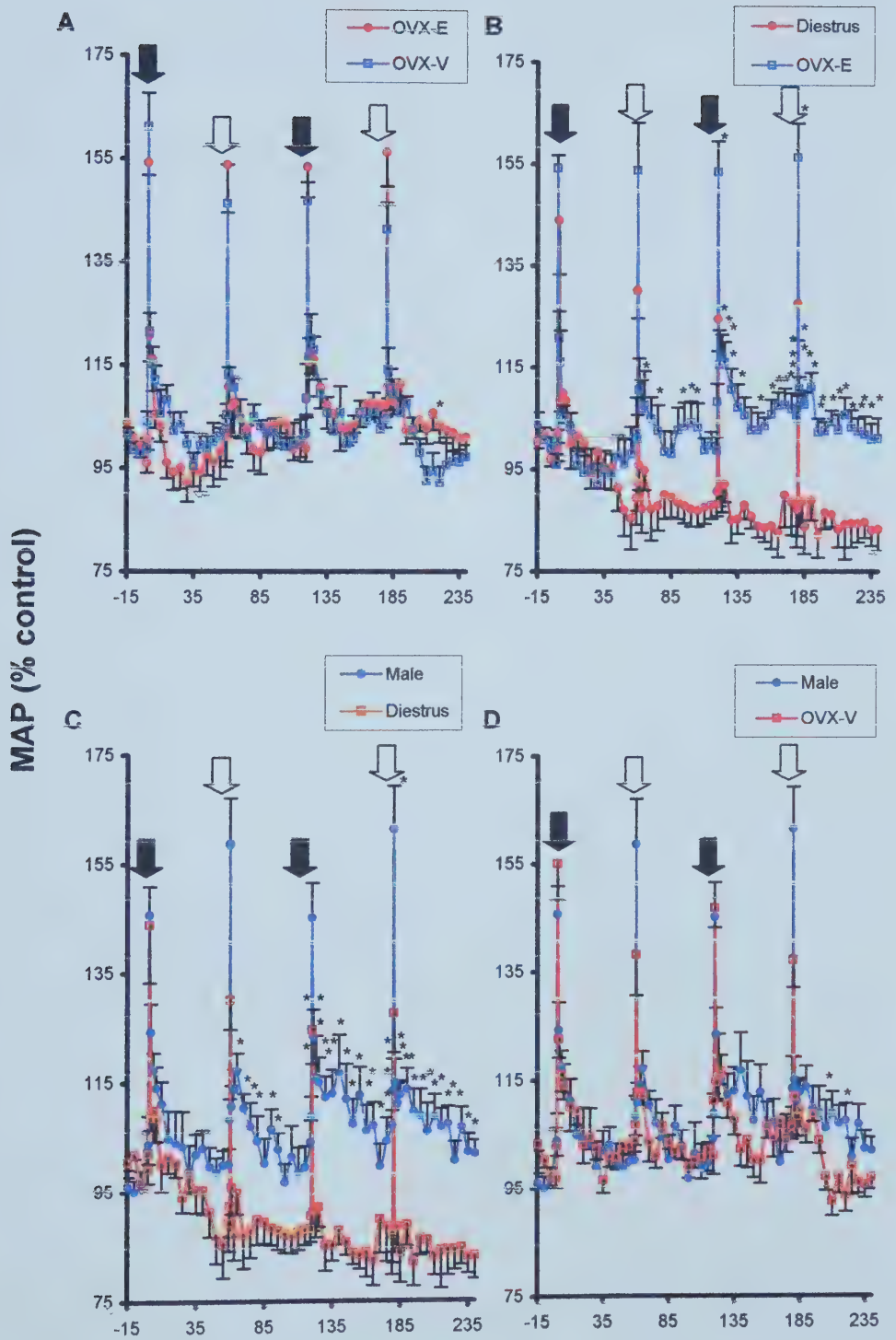








Figure 8: Comparisons of MAP between pairs of restrained animals groups. Comparisons of mean arterial pressure (MAP) between A) OVX-V (open boxes) and OVX-E (filled circles), B) OVX-E (open boxes) and diestrus (filled circles), C) diestrus (open boxes) and male (filled circles), and D) OVX-V (open boxes) and male (filled circles) rats during restraint stress. Data are calculated as a percentage of baseline, and are expressed as mean  $\pm$  SEM. Black arrows indicate the time at which rats were put into the restraint tube, white arrows indicate the time at which rats were taken out of the restraint tube. \* denotes significance,  $p < 0.05$  (ANOVA).  $n = 8$  (male),  $n = 7$  (OVX-V),  $n = 7$  (OVX-E),  $n = 5$  (diestrus).

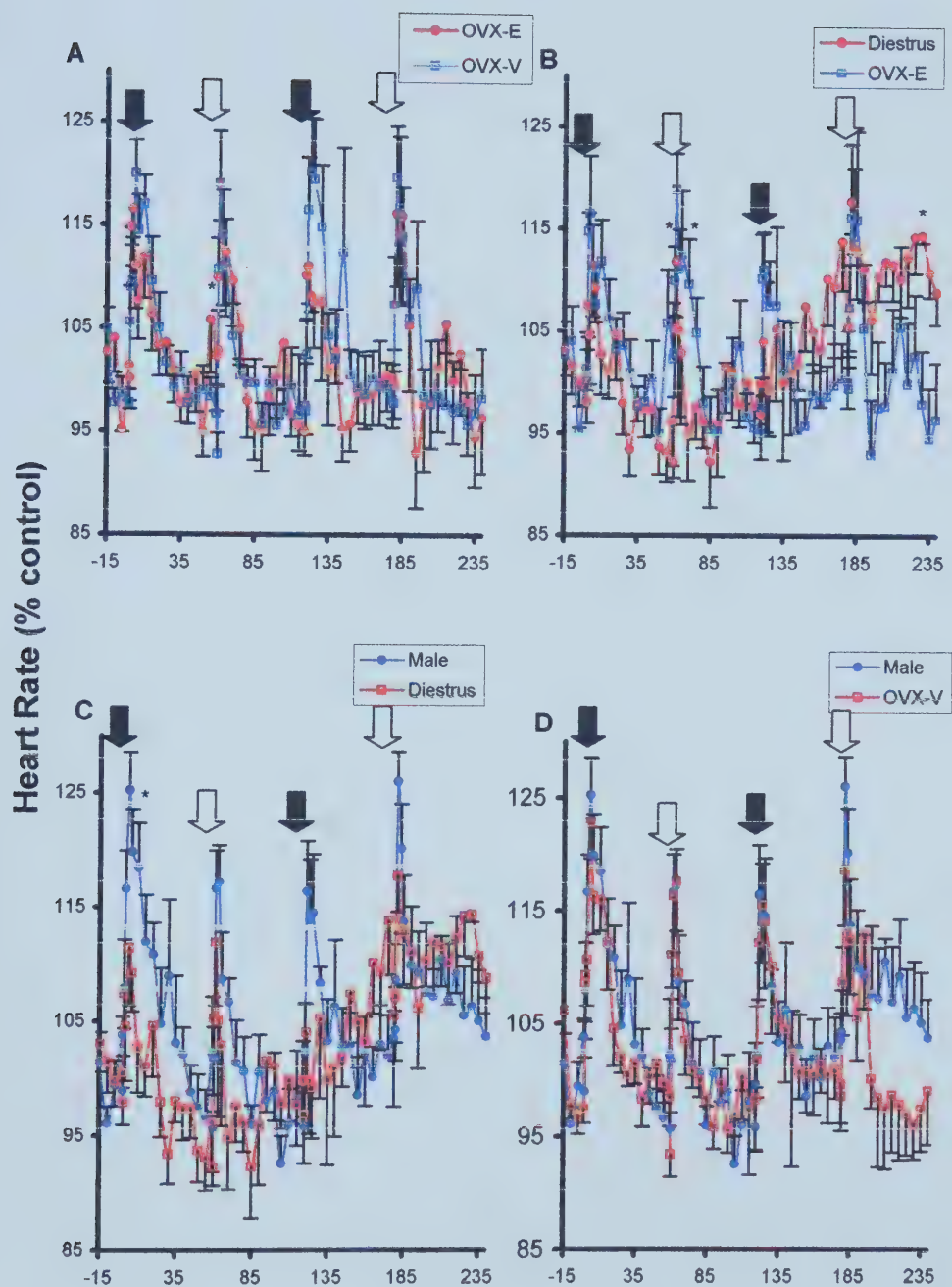


الحمد لله الذي جعلنا من  
أهل البيت من آل الله  
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والعاقبة الصالحين

والسلام على من  
آتاه الله الدين  
والعاقبة الصالحين  
والسلام على من



Figure 9: Comparisons of HR between pairs of restrained animals groups. Comparisons of heart rate (HR) between A) OVX-V (open boxes) and OVX-E (filled circles), B) OVX-E (open boxes) and diestrus (filled circles), C) diestrus (open boxes) and male (filled circles), and D) OVX-V (open boxes) and male (filled circles) rats during restraint stress. Data are calculated as a percentage of baseline, and are expressed as mean  $\pm$  SEM. Black arrows indicate the time at which rats were put into the restraint tube, white arrows indicate the time at which rats were taken out of the restraint tube. \* denotes significance,  $p < 0.05$  (ANOVA).  $n = 8$  (male),  $n = 7$  (OVX-V),  $n = 7$  (OVX-E),  $n = 5$  (diestrus).





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Figure 10: Measurements of MAP and HR in long-term treated animals. A. Recording of mean arterial pressure (MAP) in restrained LTV (open boxes) and LTE (filled circles) rats. B. Recordings of heart rate (HR) in restrained LTV (open boxes), LTE (filled circles) rats. Data are calculated as a percentage of baseline, and are expressed as mean  $\pm$  SEM. Black arrows indicate time at which rats were put into the restraint tube, white arrows indicate time at which rats were taken out of the restraint tube. \* denotes statistical significance,  $p < 0.05$  (Student's t-test). MAP:  $n = 8$  (LTV),  $n = 8$  (LTE). HR:  $n = 7$  (LTV),  $n = 6$  (LTE).

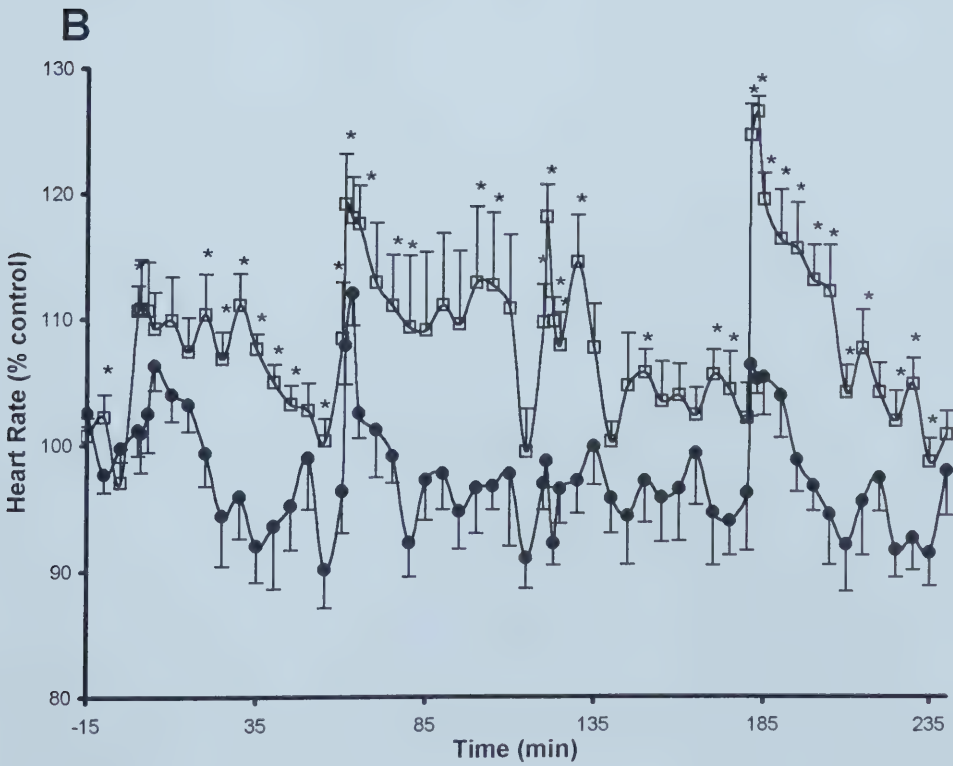
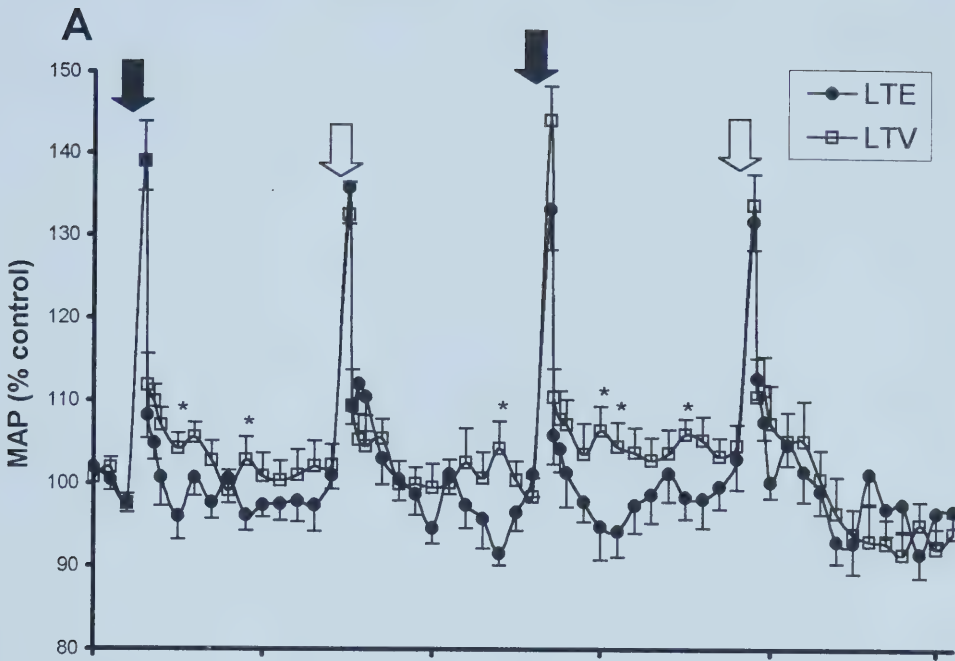






Figure 11: TH mRNA levels in the LC of unrestrained and restrained rats. Expression of tyrosine hydroxylase (TH) mRNA in the locus coeruleus (LC) of A) unrestrained and B) restrained OVX-V (open bar), OVX-E (filled bar), diestrus (diagonal lines), and male (horizontal lines) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance from diestrus rats,  $p < 0.05$  (ANOVA). *n*-values are located within the corresponding bars.

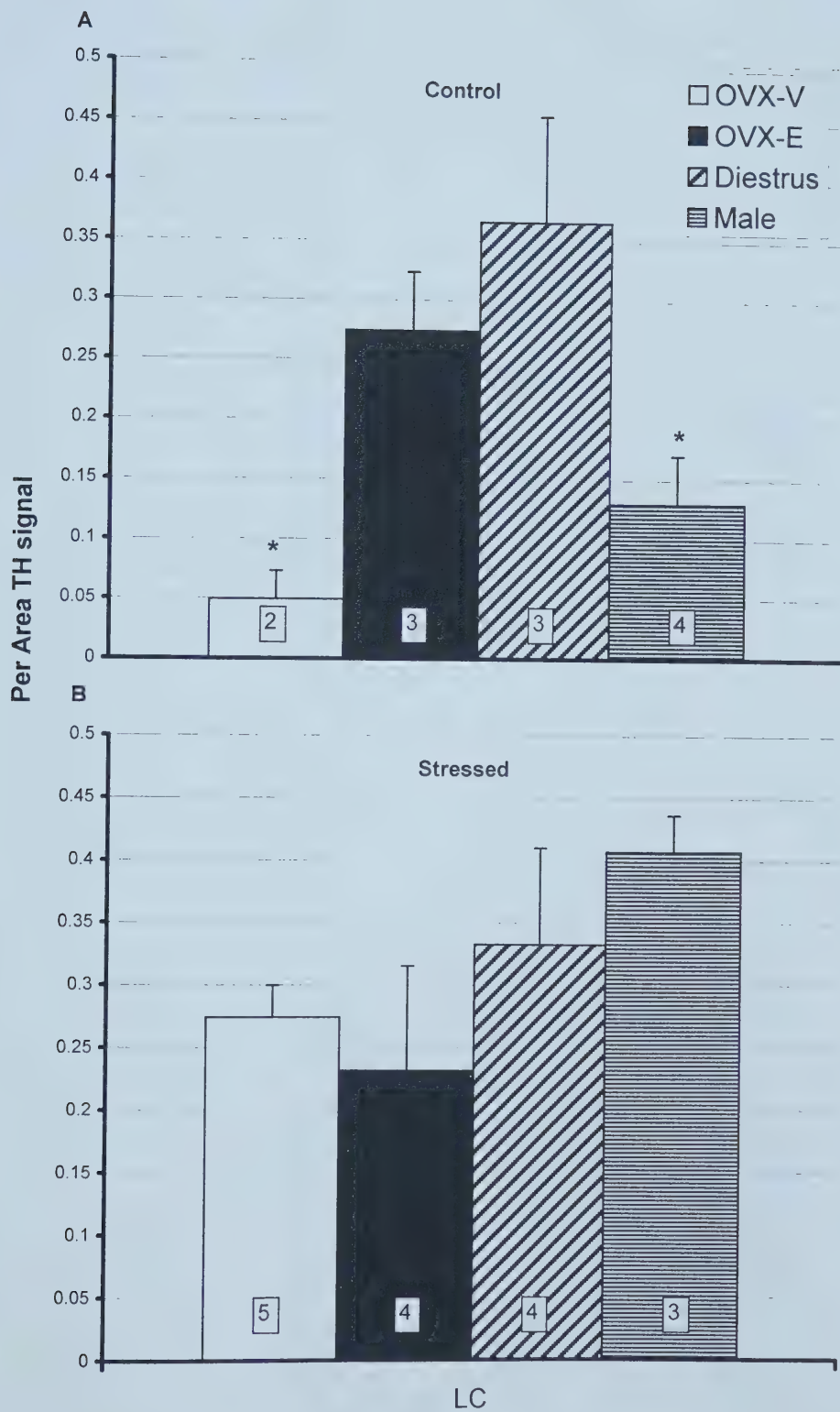








Figure 12: Comparison of TH mRNA levels in the LC between restrained and unrestrained rats. Expression of tyrosine hydroxylase (TH) mRNA in the locus coeruleus (LC) of A) unrestrained OVX-V (open bar) vs. restrained OVX-V (closed bar) rats B) unrestrained OVX-E (open bar) vs. restrained OVX-E (closed bar) rats C) unrestrained diestrus (open bar) vs. restrained diestrus (closed bar) rats and D) unrestrained male (open bar) vs. restrained male (closed bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test).  $n$ -values are the same as in Figure 11.

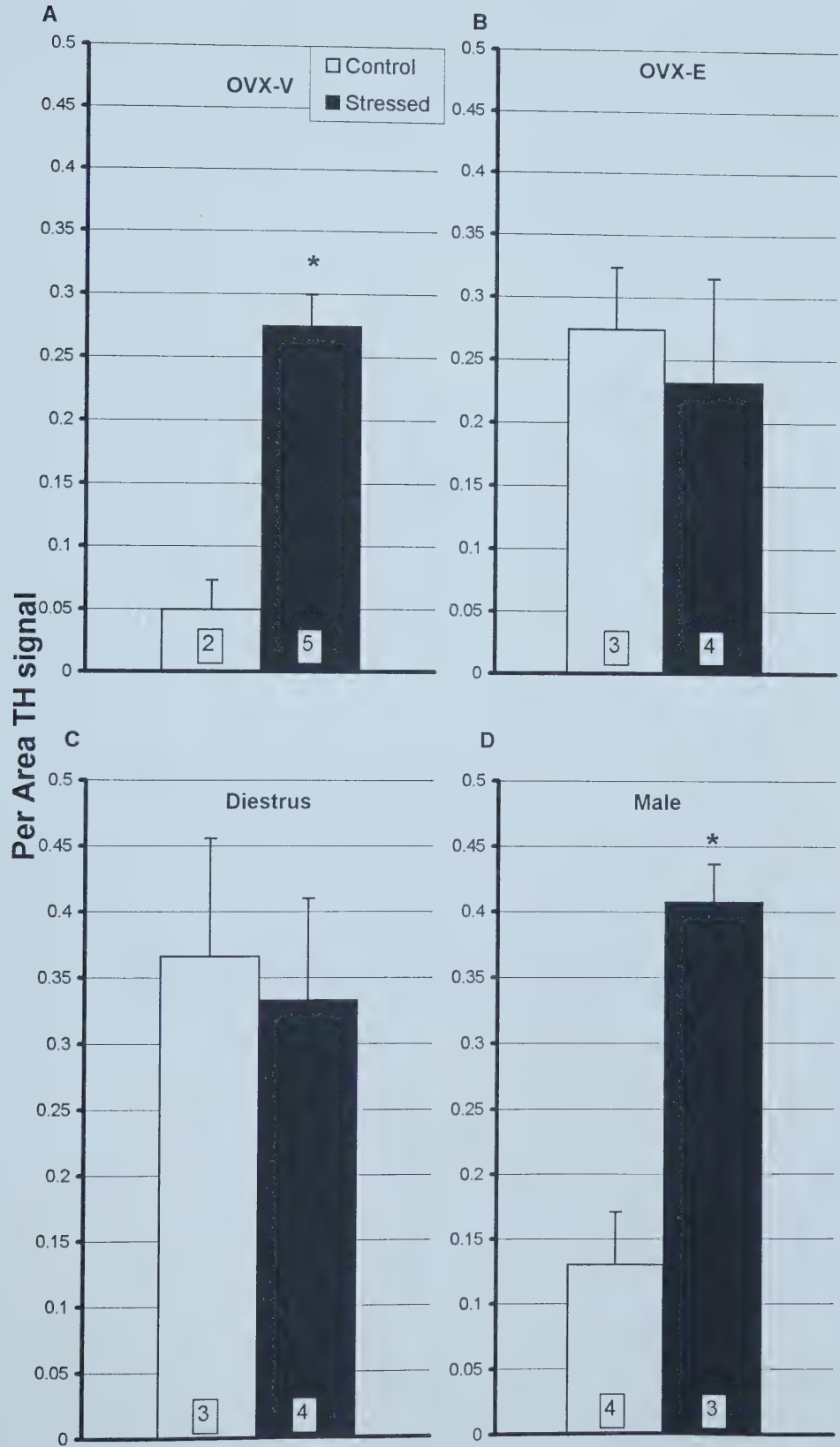






Figure 13: Expression of TH mRNA in the locus coeruleus (LC) of A) unrestrained and B) restrained male rats. The LC is located to the right of the 4<sup>th</sup> ventricle. Bars represent 25  $\mu$ m.

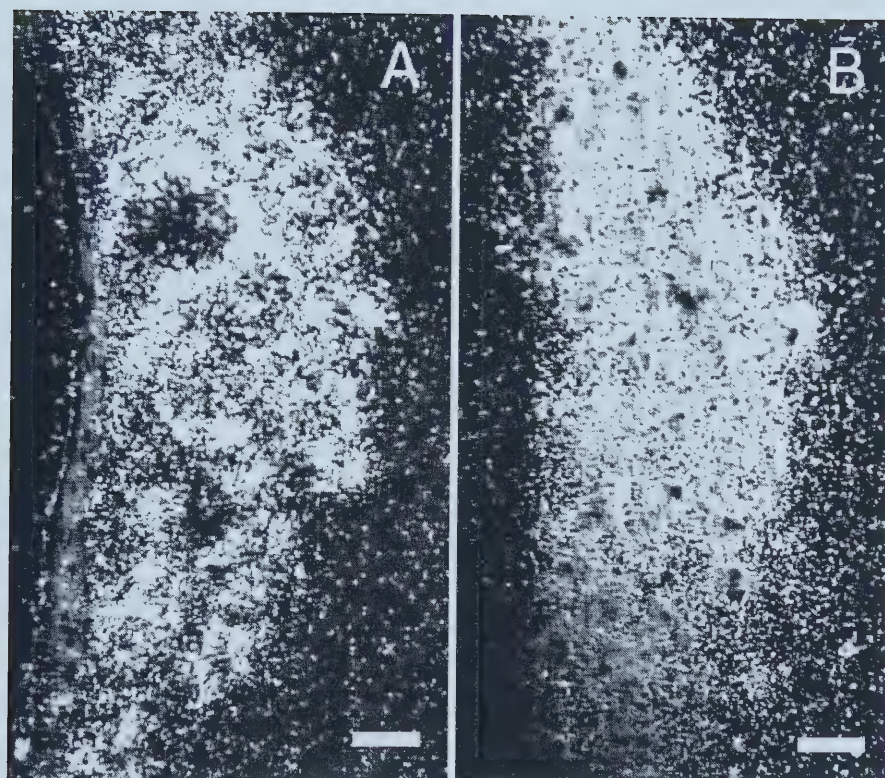








Figure 14: Nitrate/nitrite concentration in the hypothalamus and brainstem of unrestrained and restrained rats. Concentration of nitrates/nitrites in the hypothalamus and brainstem of A) unrestrained and B) restrained OVX-V (open bar), OVX-E (filled bar), diestrus (diagonal lines), and male (horizontal lines) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance from OVX-E rats, + indicates statistical significance from male rats,  $p < 0.05$  (ANOVA). *n*-values are located within the corresponding bars.

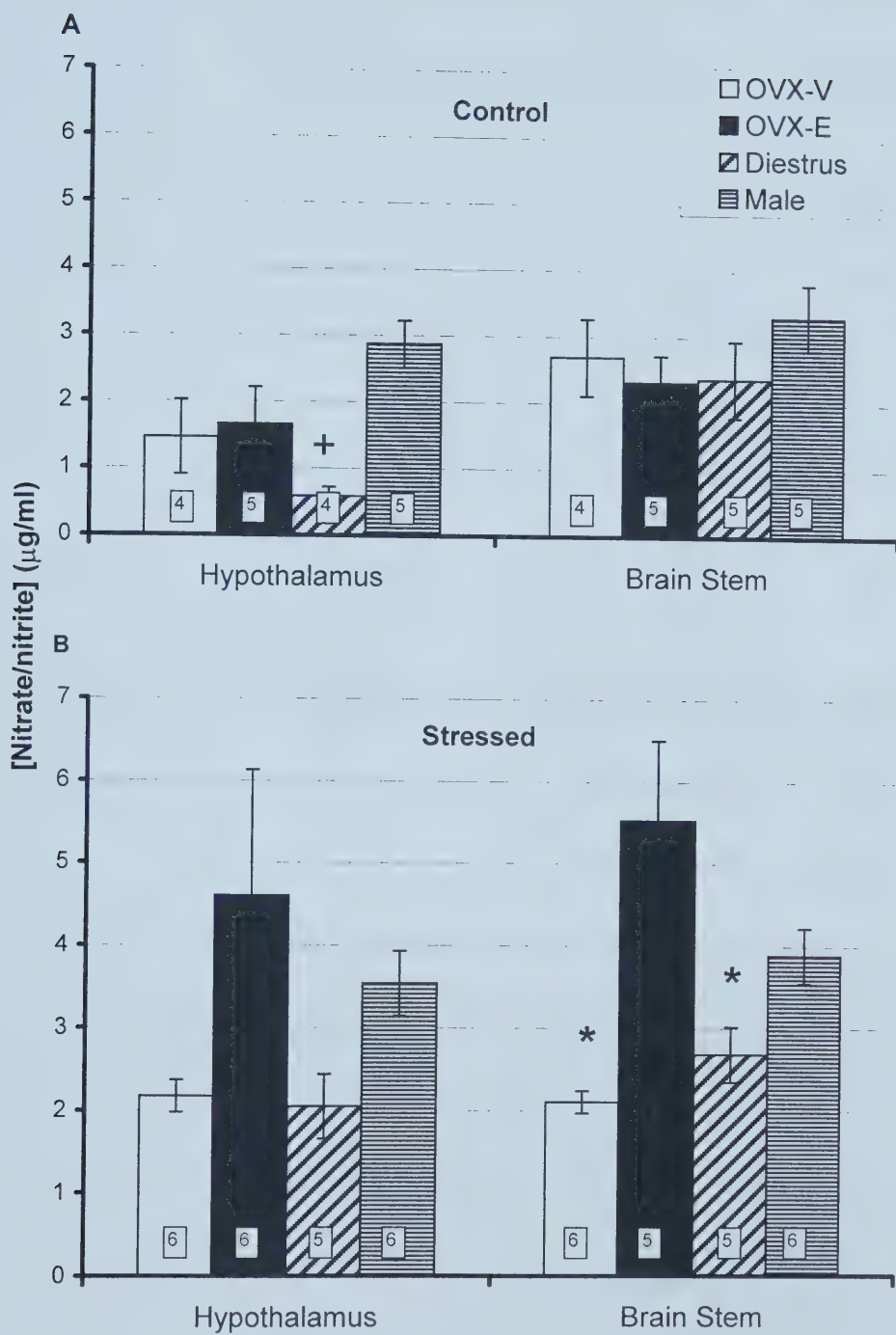
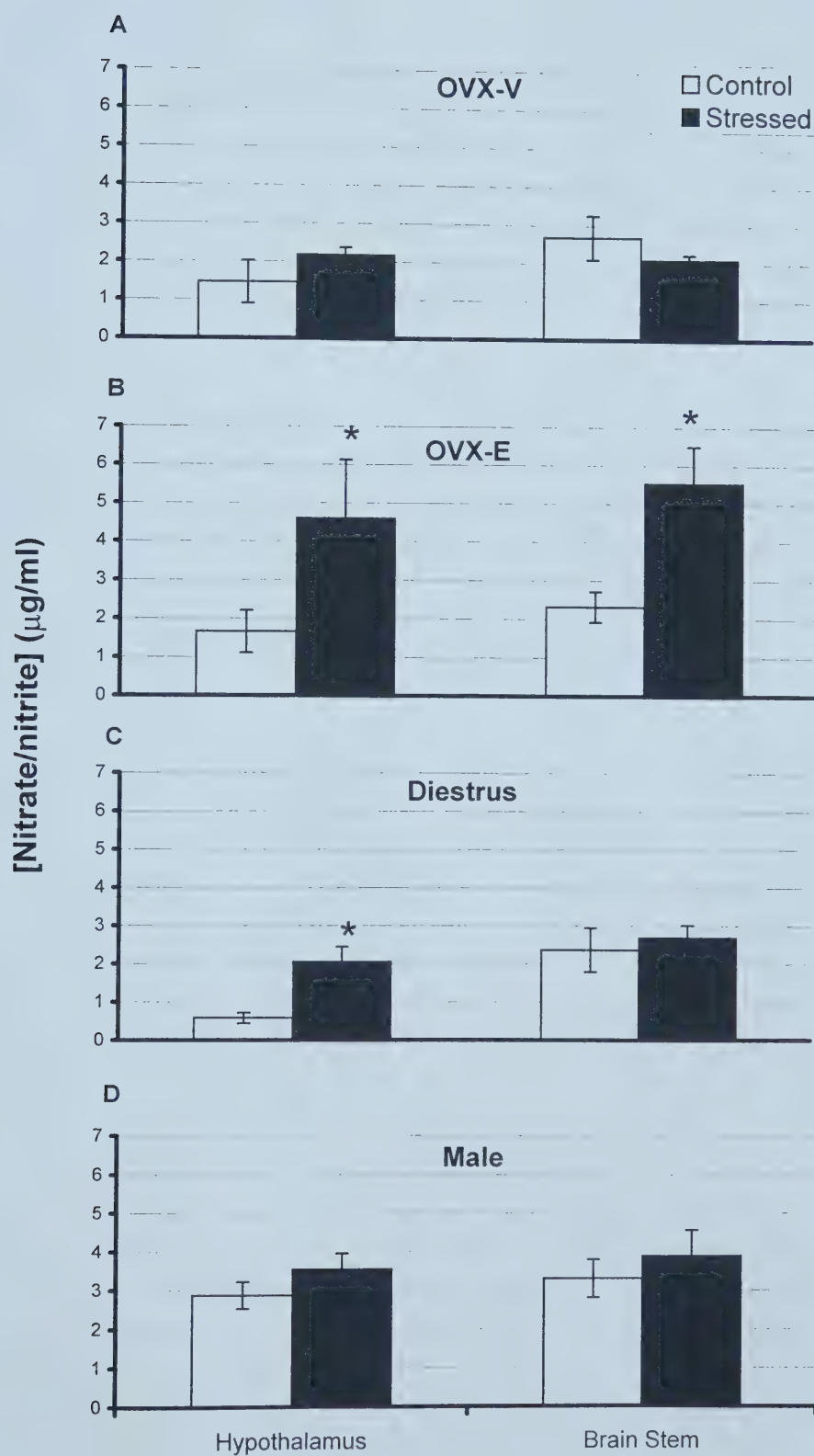






Figure 15: Comparison of nitrate/nitrite concentration in the hypothalamus and brainstem between restrained and unrestrained rats. Comparison of nitrate/nitrite concentration in the hypothalamus and brainstem of A) unrestrained OVX-V (open bar) vs. restrained OVX-V (closed bar) rats, B) unrestrained OVX-E (open bar) vs. restrained OVX-E (closed bar) rats C) unrestrained diestrus (open bar) vs. restrained diestrus (closed bar) rats and D) unrestrained male (open bar) vs. restrained male (closed bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test).  $n$ -values are the same as in Figure 14.





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Figure 16: Comparison of nitrate/nitrite concentration in the hypothalamus and brainstem of restrained long-term treated animals, LTV (open bar) vs. LTE (filled bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test). *n*-values are located within the corresponding bars.

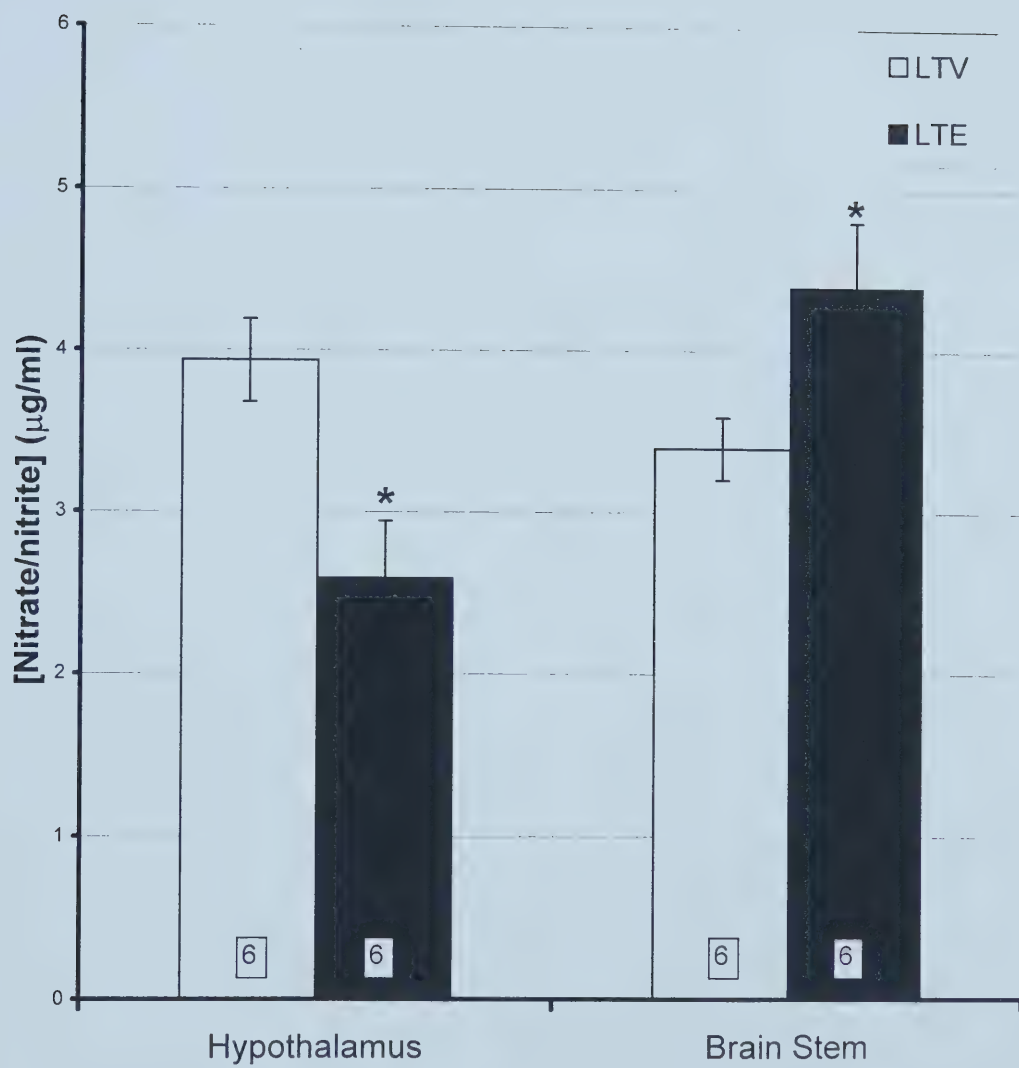






Figure 17: Numbers of NADPHd stained neurons in the PVN of unrestrained and restrained rats. Measurement of the number of NADPHd positive neurons in the subdivisions of the paraventricular nucleus (PVN) of A) unrestrained and B) restrained OVX-V (open bar), OVX-E (filled bar), diestrus (diagonal lines), and male (horizontal lines) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance from all other groups, + indicates statistical significance from male rats, # indicates significance from diestrus,  $p < 0.05$  (ANOVA). Unless otherwise noted within bars,  $n$ -values for all groups are located within the respective rostral subdivision measurement bars. Abbreviations: pm, posterior magnocellular subdivision; dp, dorsal parvocellular subdivision; mpv, ventral medial parvocellular subdivision; mpd, dorsal medial parvocellular subdivision; lp, lateral parvocellular subdivision; mp, medial parvocellular subdivision.

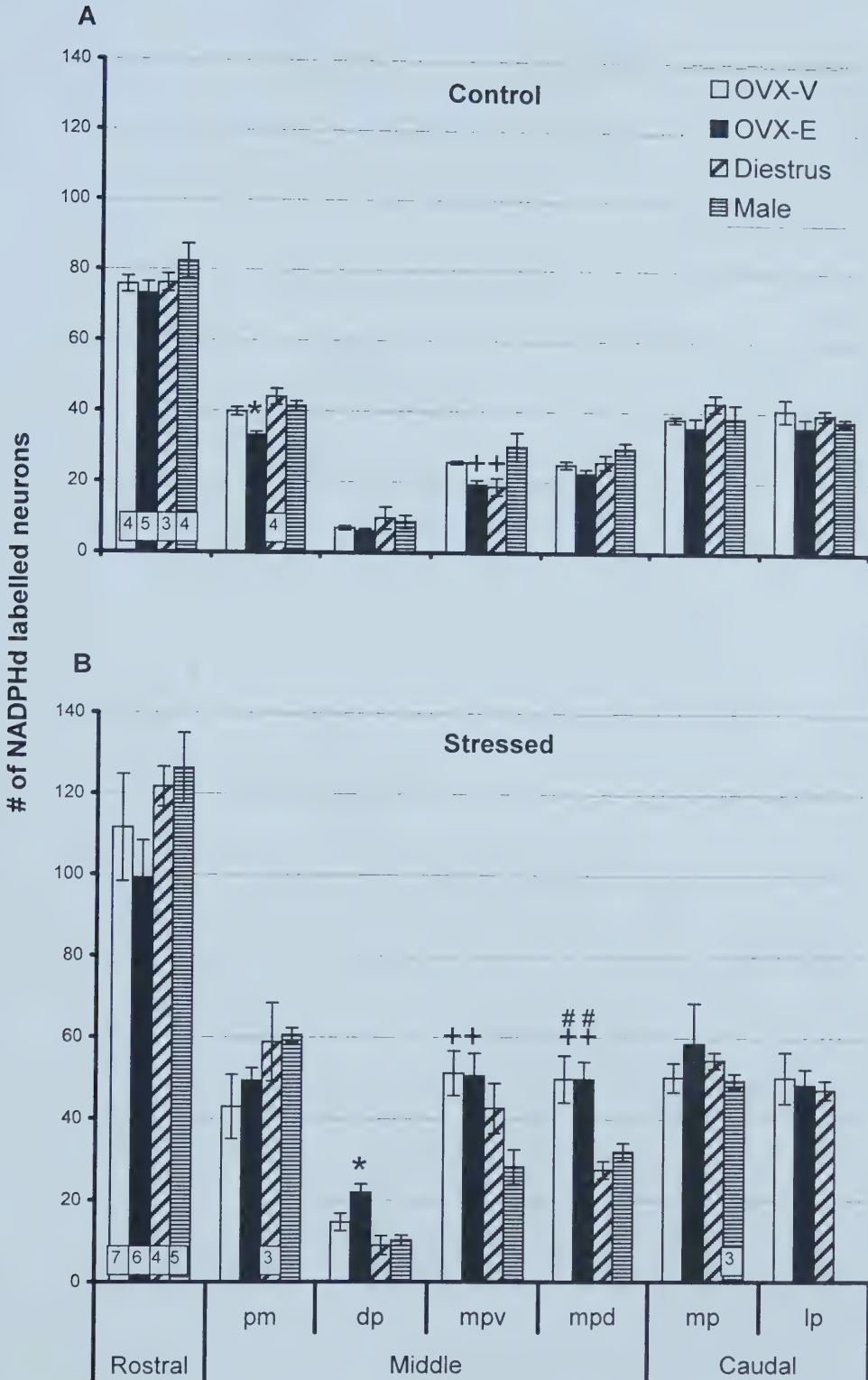








Figure 18: Comparison of the number of NADPHd stained neurons in the PVN between restrained and unrestrained rats. Comparison of the number of NADPHd stained neurons in the paraventricular nucleus (PVN) of A) unrestrained OVX-V (open bar) vs. restrained OVX-V (closed bar) rats, B) unrestrained OVX-E (open bar) vs. restrained OVX-E (closed bar) rats, C) unrestrained male (open bar) vs. restrained male (closed bar) rats, and D) unrestrained diestrus (open bar) vs. restrained diestrus (closed bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test). P-values approaching significance are shown. *n*-values are the same as in Figure 17. Abbreviations: pm, posterior magnocellular subdivision; dp, dorsal parvocellular subdivision; mpv, ventral medial parvocellular subdivision; mpd, dorsal medial parvocellular subdivision; lp, lateral parvocellular subdivision; mp, medial parvocellular subdivision; R, rostral; M, middle; C, caudal







Figure 19: Comparison of number of NADPHd stained neurons in the PVN of restrained long-term treated animals, LTV (open bar) vs. LTE (filled bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test). *n*-values are located within the respective rostral subdivision measurement bars. Abbreviations: pm, posterior magnocellular subdivision; dp, dorsal parvocellular subdivision; mpv, ventral medial parvocellular subdivision; mpd, dorsal medial parvocellular subdivision; lp, lateral parvocellular subdivision; mp, medial parvocellular subdivision.

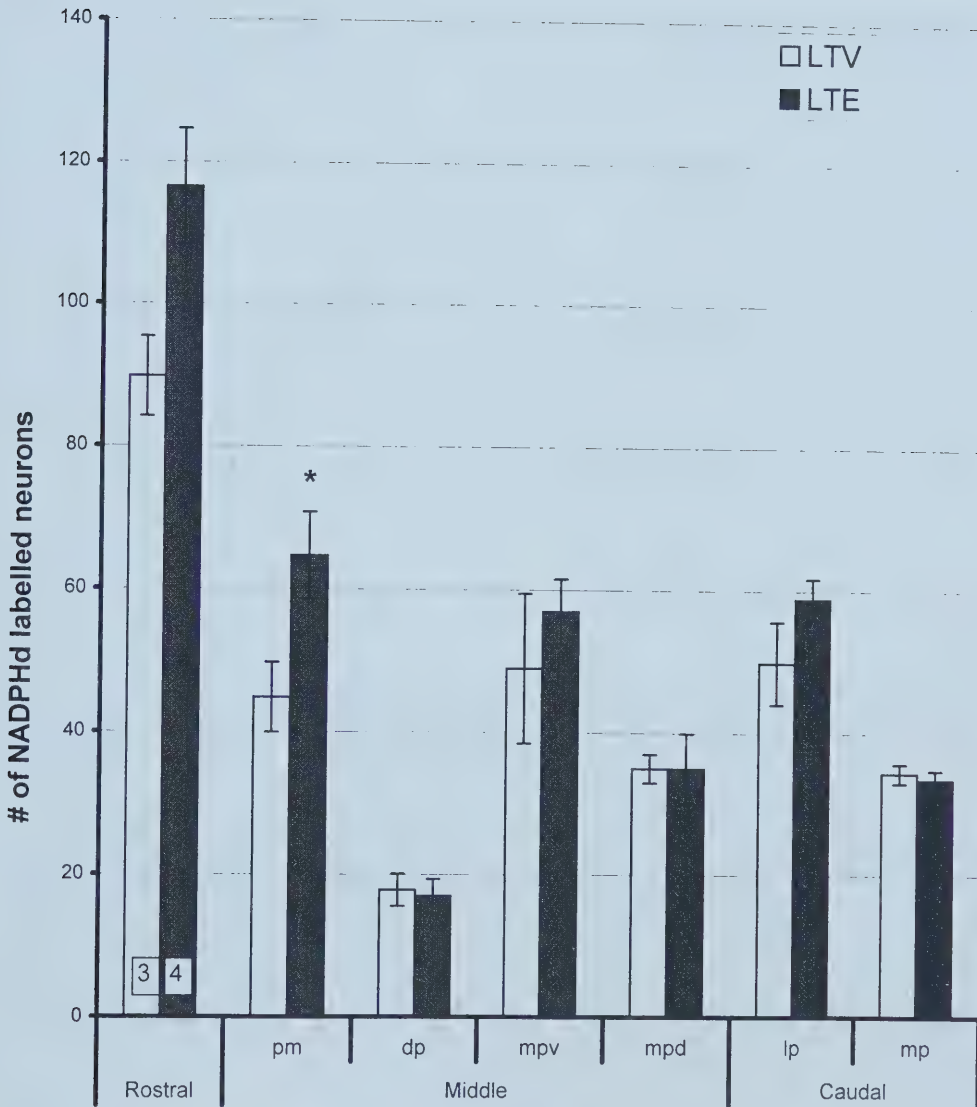






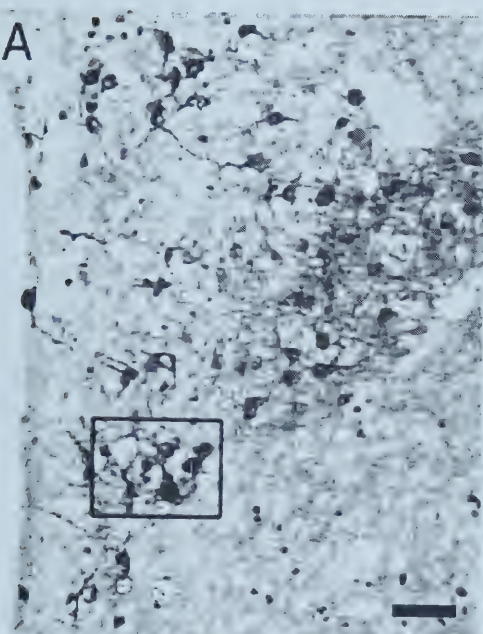


Figure 20: Expression of NADPHd in the PVN. Expression of NADPHd in the paraventricular nucleus (PVN) of A, C) unrestrained OVX-E rats and B, D) restrained OVX-E rats.. The areas in the rectangles in A and B are shown at higher magnification in C and D respectively. Bars in A and B represent 50 $\mu$ m. Bars in C and D represent 10 $\mu$ m.

Control

Stressed

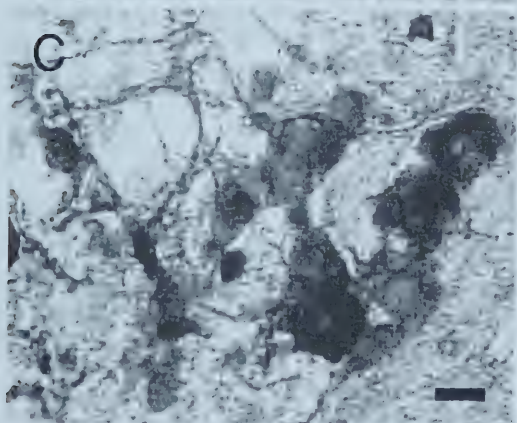
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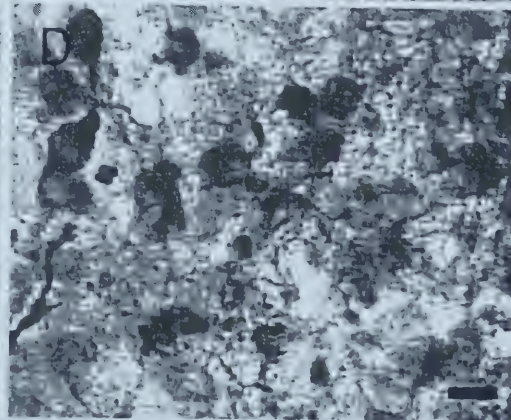
B



C



D





)

Figure 21: CRF mRNA levels in the middle PVN of unrestrained and restrained rats. Expression of corticotropin releasing factor (CRF) mRNA in the middle paraventricular nucleus (PVN) of A) unrestrained and B) restrained OVX-V (open bar), OVX-E (filled bar), diestrus (diagonal lines), and male (horizontal lines) rats. Data are expressed as mean  $\pm$  SEM. + indicates statistical significance from male rats,  $p < 0.05$  (ANOVA). *n*-values are located within the respective pm subdivision measurement bars. Abbreviations: pm, posterior magnocellular subdivision; dp, dorsal parvocellular subdivision; mpv, ventral medial parvocellular subdivision; mpd, dorsal medial parvocellular subdivision.

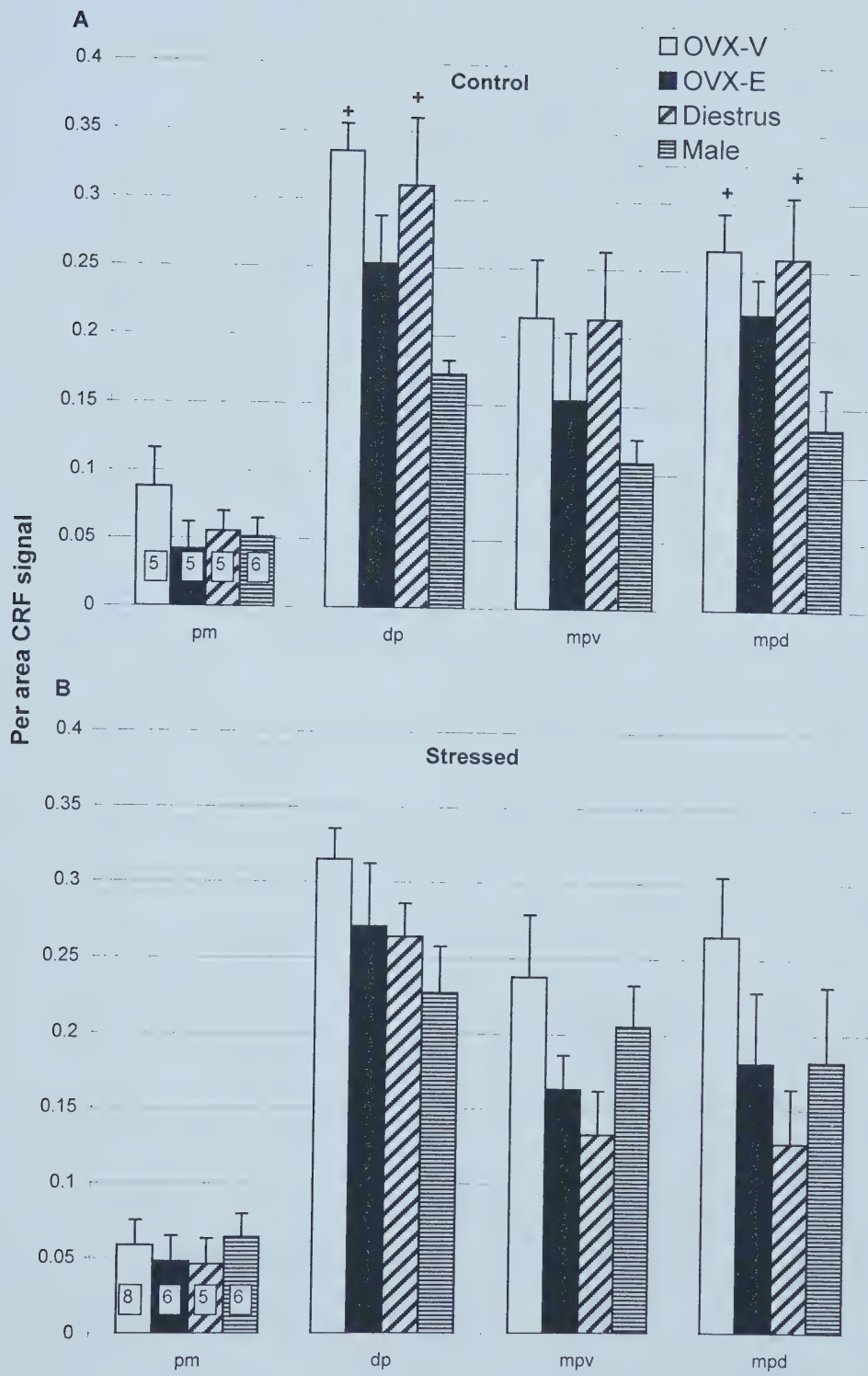








Figure 22: Comparison of CRF mRNA levels in the PVN between restrained and unrestrained rats. Expression of corticotropin releasing factor (CRF) mRNA in the middle paraventricular nucleus (PVN) of A) unrestrained OVX-V (open bar) vs. restrained OVX-V (closed bar) rats, B) unrestrained OVX-E (open bar) vs. restrained OVX-E (closed bar) rats, C) unrestrained diestrus (open bar) vs. restrained diestrus (closed bar) rats, and D) unrestrained male (open bar) vs. restrained male (closed bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test). P-values approaching significance are shown.. *n*-values are as located in Figure 21.

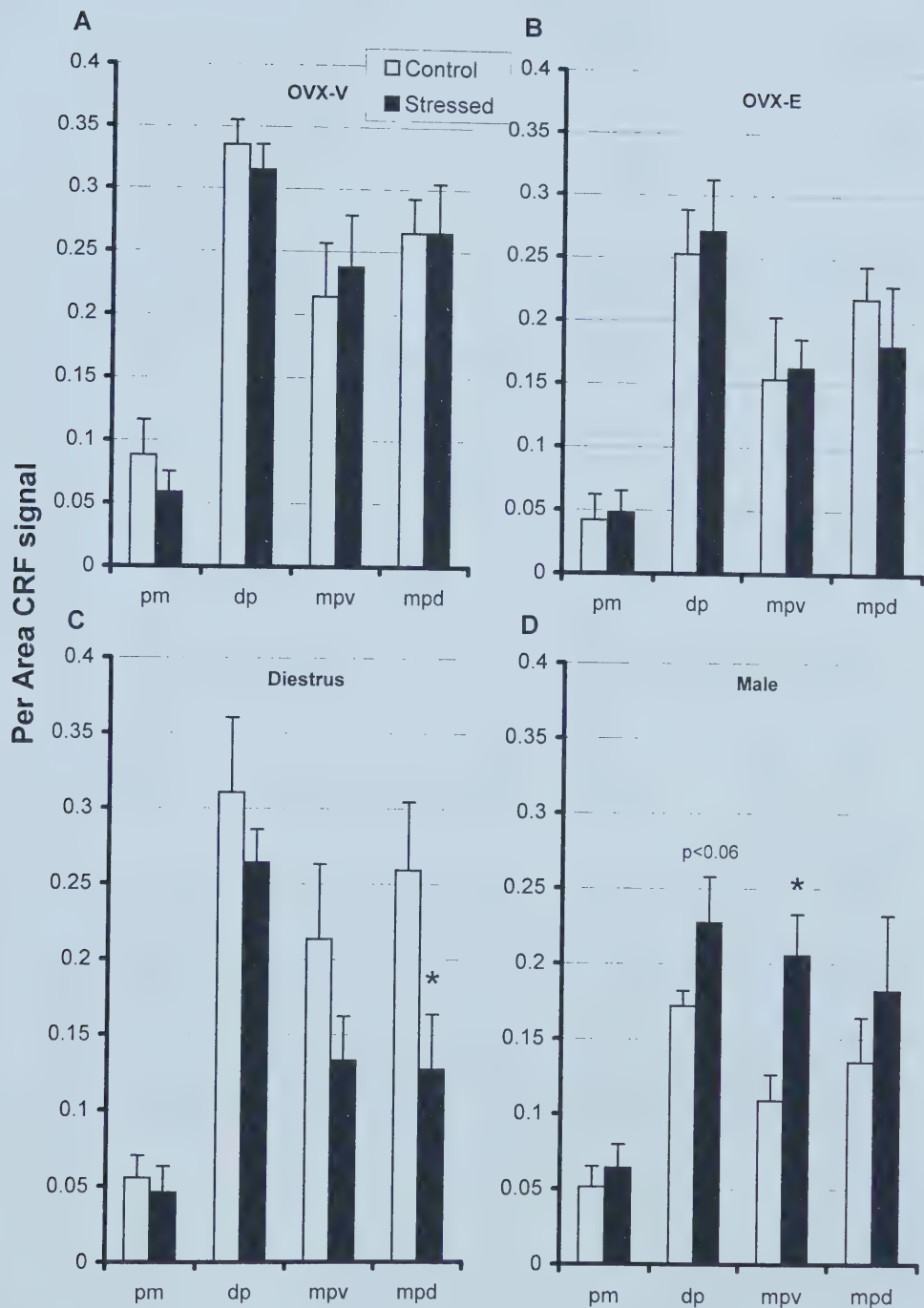
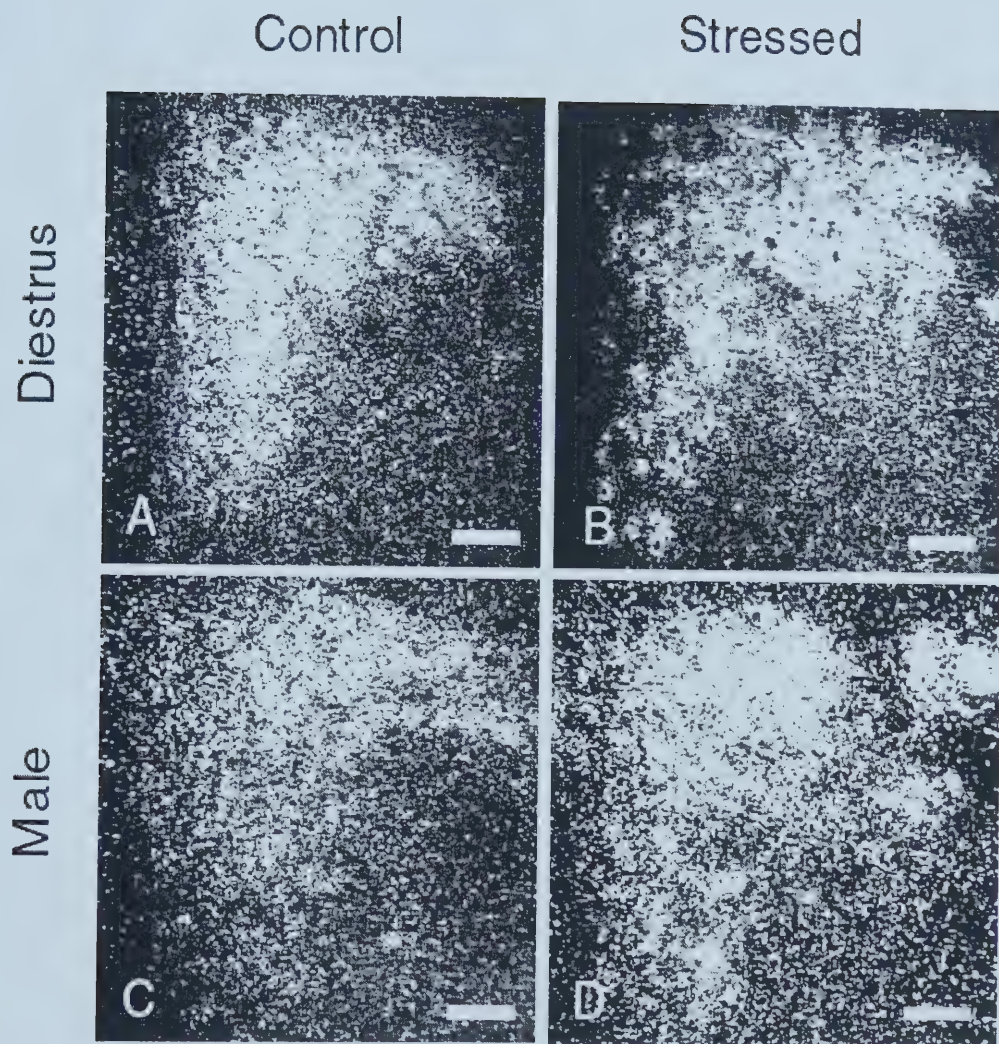






Figure 23: Expression of CRF mRNA in the PVN. Expression of corticotropin releasing hormone (CRF) mRNA in the middle paraventricular nucleus (PVN) of A) unrestrained diestrus rats, B) restrained diestrus rats, C) unrestrained male rats, and D) restrained male rats. Bars represent 50 $\mu$ m.





1  
2

4



Figure 24: Plasma ACTH levels in unrestrained and restrained rats. Expression of adrenocorticotropin hormone (ACTH) in the plasma of A) unrestrained and B) restrained OVX-V (open bar), OVX-E (filled bar), diestrus (diagonal lines), and male (horizontal lines) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance from all other groups,  $p < 0.05$  (ANOVA). *n*-values are located within the corresponding bars.

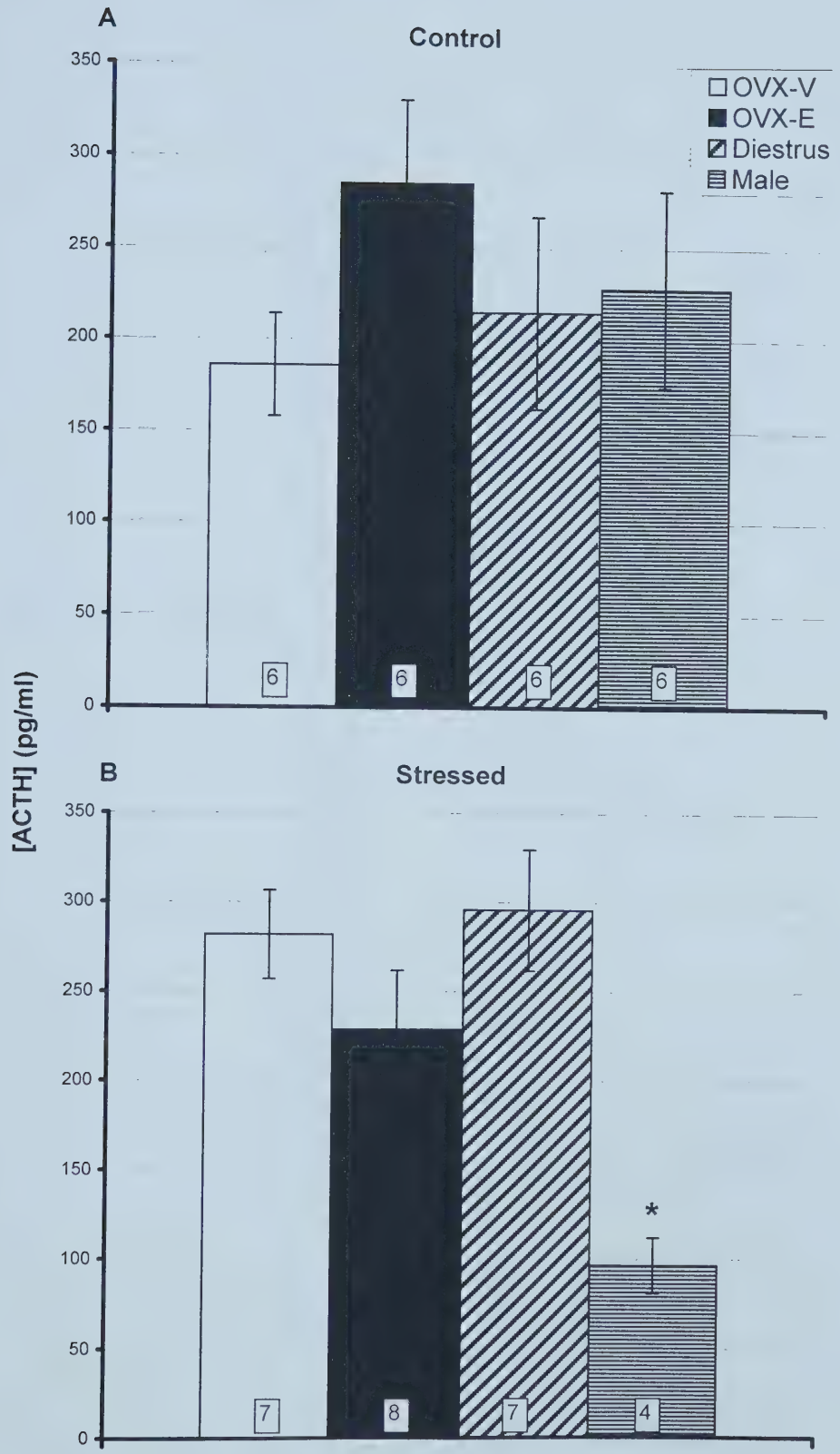






Figure 25: Comparison of ACTH plasma concentration between restrained and unrestrained rats. Expression of adrenocorticotropin hormone (ACTH) in the plasma of A) unrestrained OVX-V (open bar) vs. restrained OVX-V (closed bar) rats, B) unrestrained OVX-E (open bar) vs. restrained OVX-E (closed bar) rats, C) unrestrained male (open bar) vs. restrained male (closed bar) rats, and D) unrestrained diestrus (open bar) vs. restrained diestrus (closed bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test).  $n$ -values are the same as in Figure 24.

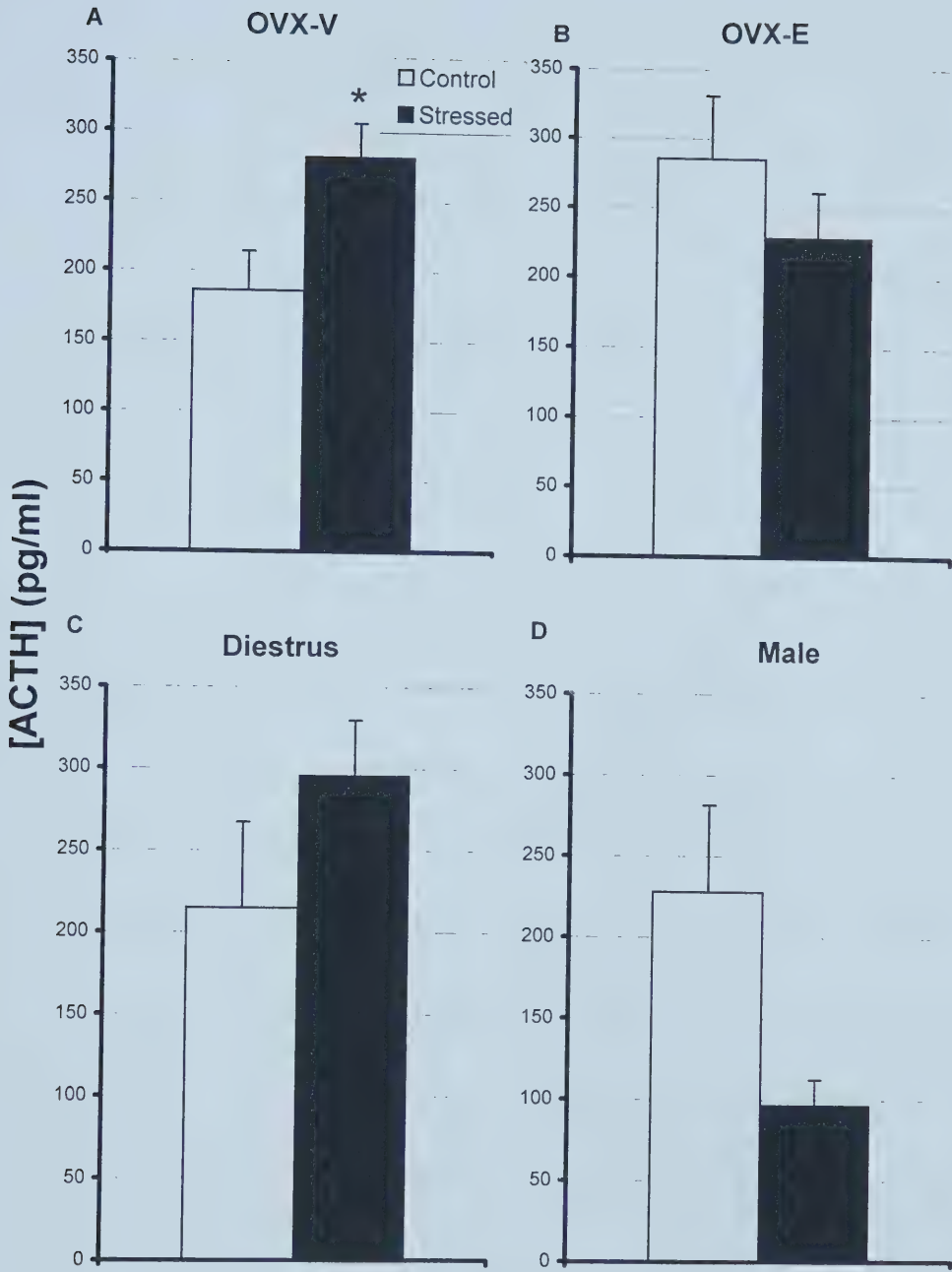








Figure 26: Comparison of ACTH plasma concentration in long-term treated animals, LTV (open bar) vs. LTE (filled bar) rats. Data are expressed as mean  $\pm$  SEM. \* indicates statistical significance,  $p < 0.05$  (Student's t-test).  $n$ -values are located within the corresponding bars.

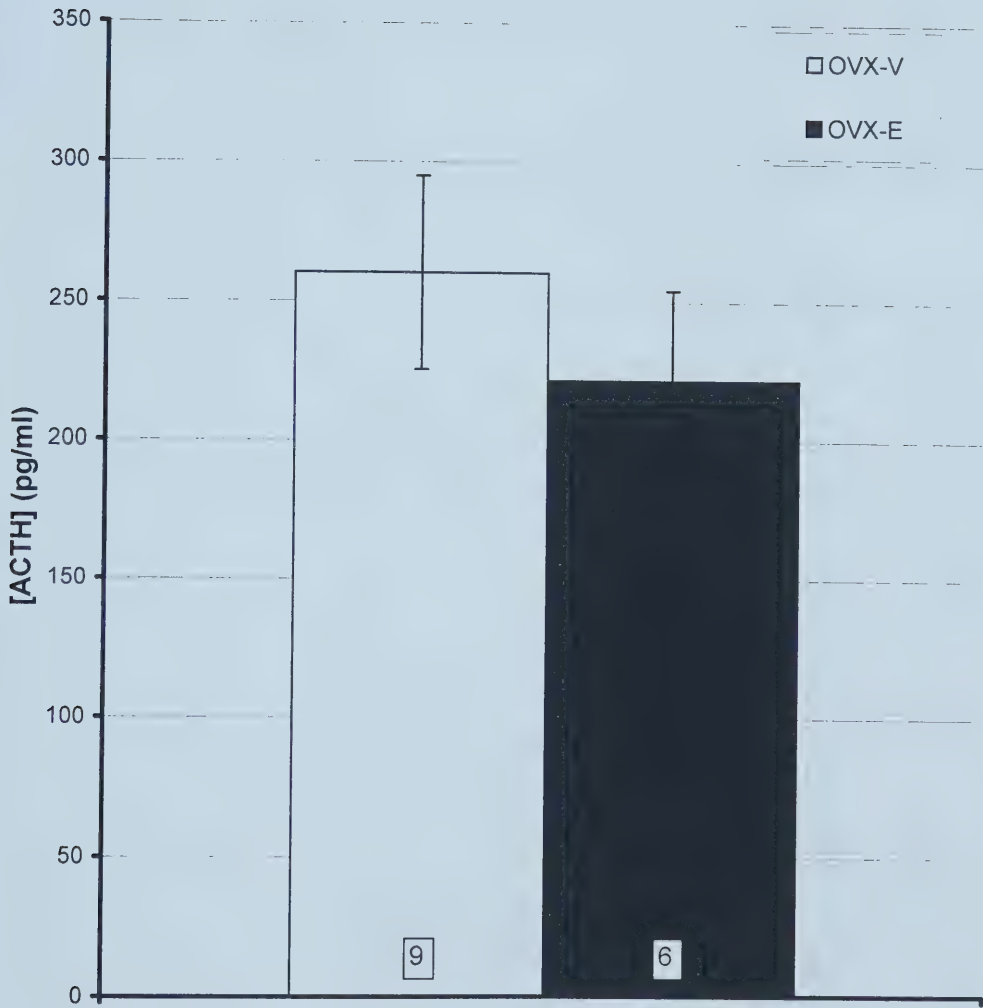
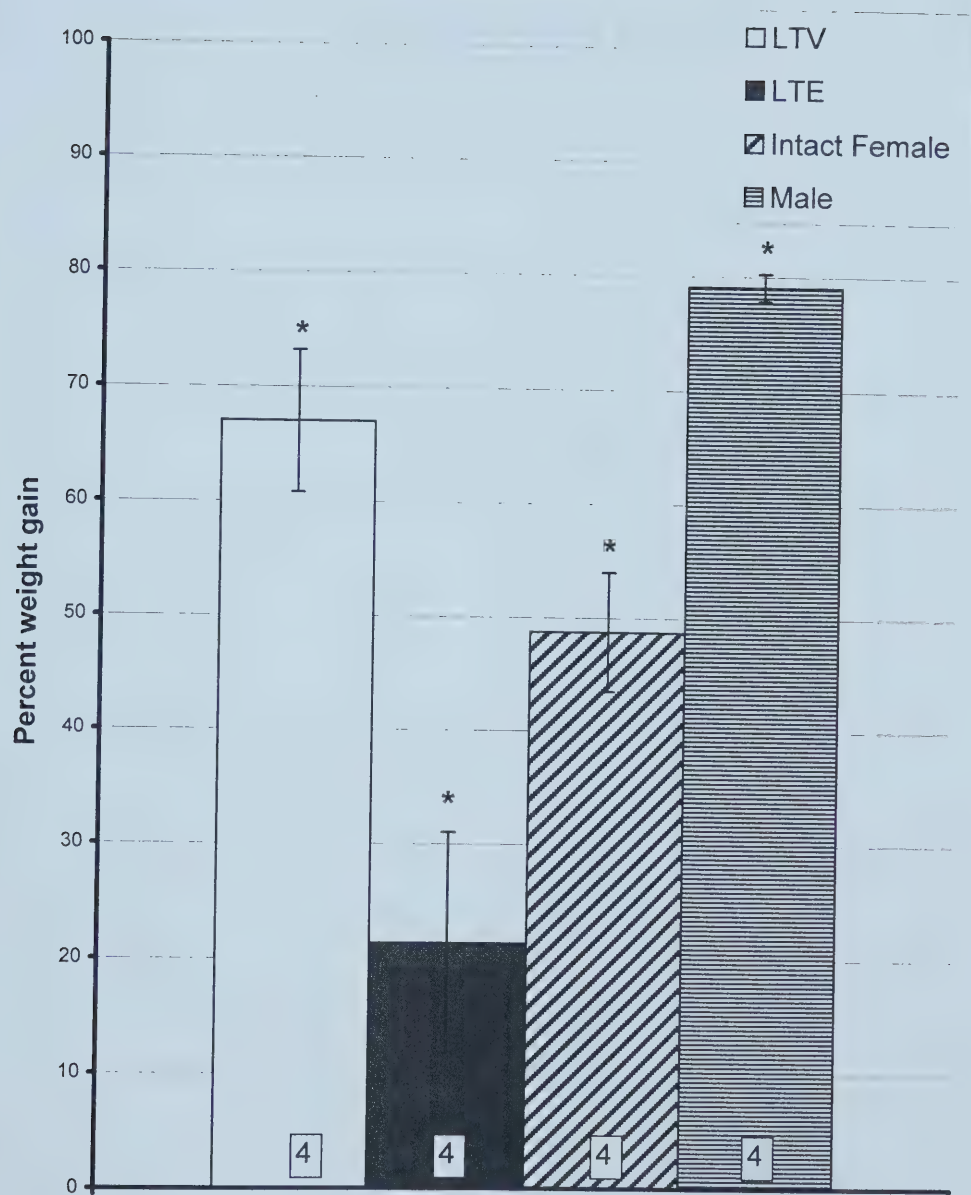






Figure 27: Weight gain exhibited over 7-weeks in OVX-V (open bar), OVX-E (filled bar), intact females (diagonal lines), and intact males (horizontal lines). \* indicates statistical significance from all other groups (ANOVA). *n*-values are located within the corresponding bars.





## **CHAPTER 4**

## **DISCUSSION**





We have shown that the MAP and HR responses to restraint in ovariectomized (OVX) rats with or without estrogen replacement are similar, but OVX animals treated with estrogen (OVX-E) appear to adapt faster to stress. These results suggest that the presence of estrogen attenuates sympathetic output upon stress. There is also an effect of the ovariectomy itself as OVX-E rats appear to have reduced efficiency of the baroreceptor reflex. When comparing the intact animal groups, males have responses similar to the OVX rats, whereas intact diestrus females exhibited responses to stressors where MAP falls below baseline and HR remains above it. These data exhibit a sexually dimorphic sympathetic response to stress. Therefore, it appears that estrogen treatment attenuated sympathetic output, and that ovariectomy and gender influenced the cardiovascular responses to restraint. Measurements of TH mRNA in the LC upon restraint stress are only increased in rats lacking estrogen indicating that estrogen may be associated with the stabilization of sympathetic output upon stress. When measuring responses of the HPA axis to restraint, decreased CRF mRNA levels in the PVN were observed due to ovariectomy and opposite reactions to stress were seen related to gender. Diestrus females showed decreased CRF mRNA levels throughout the PVN, and males showed increased CRF mRNA levels throughout the PVN. When measuring central NO concentrations upon restraint, increases are seen in animals with estrogen, but not males. Estrogen appears to be involved in the upregulation of central NO, and there is a sexual dimorphic effect. Increased numbers of NO-producing neurons in the PVN upon restraint are



seen in all female groups indicating another sexually dimorphic response to stress. Our results have shown that estrogen replacement, ovariectomy and gender all influence sympathetic output, central NO production, and HPA axis activity.

#### **4.1. MAP and HR controls**

While there were no significant changes in unrestrained control MAP or HR, there is a trend that the control male HR is increasing above the rest. This increase in males may be due to a delayed response to handling and connection of the pressure transducer 30 minutes before recording. This increase may help to explain why men exhibit higher cardiovascular responsiveness to mental stress than women with a high endogenous estrogen concentration (Hastrup and Light, 1984).

#### **4.2. MAP and HR during restraint**

Upon comparisons between stressed animals and their respective controls, differences were seen according to estrogen treatment, ovariectomy, and sex of the animals. After the initial stress, estrogen treated animals responded to stressors more efficiently via more rapid attenuation of MAP and HR response to stress in comparison to vehicle treated animals. They also showed increased adaptation to the stressors via attenuated cardiovascular



response to the stressor upon each stimulus. Our data agree with past data that women with a higher estrogen plasma concentration have attenuated cardiovascular responsiveness to mental stress compared to women lower estrogen plasma concentration (Hastrup and Light, 1984). There is much data suggesting that estrogen can decrease vasoconstriction peripherally (Sudhir et al. 1997; Reviewed in Ruehlmann and Mann, 2000) and decrease cardiac output (Raddino et al., 1986), but the data offered here exhibit changes in both blood pressure and heart rate. Thus, our data begin to describe the effects of estrogen on central pathways that mediate sympathetic output.

Comparisons between the animal groups with estrogen have revealed MAP and HR differences that are likely due to the ovariectomy. Where the MAP and HR of stressed OVX-E rats remain approximately 5-10 % above controls after the initial increase due to stress, the MAP of diestrus rats falls approximately 15-20 % below controls after the initial increase and the HR steadily increases above controls. These observations suggest that the baroreceptor reflex may be strongly affected in stressed diestrus females, and attenuated in OVX-E rats. Therefore, it appears that the presence of all ovarian hormones is important in the efficiency of the baroreceptor reflex. Previous data have shown that in OVX rats given  $17\beta$ -estradiol, the baroreceptor reflex was augmented to a level similar to or higher than sham operated rats, indicating that estrogen alone facilitates baroreflex function (El-Mas and Abdel-Rahman, 1998; Mohamed et al., 1999).



Differences were seen when comparing the MAP and HR responses to restraint in intact females and intact males. Males responded to the stress paradigm similarly as both ovariectomized groups, whereas the diestrus females responded as described above. Males adapt to stressors by reducing the time that MAP and HR were higher than unrestrained controls after the initial increase. Intact females have a strong baroreceptor reflex in response to stressors exhibiting MAP approximately 15-20 % below unrestrained control levels and HR approximately 15-20 % above unrestrained controls after the initial increase. Therefore, there appears to be a sexually dimorphic cardiovascular response to restraint. There appears to be an amplified baroreceptor reflex in the females, where it is not as efficient in the males. These observations appear contradictory to published works where males exhibited a more effective baroreflex than females in response to intravenous injections of phenylephrine or nitroprusside to increase and decrease blood pressure respectively (El-Mas and Abdel-Rahman, 1998; Abdel-Rahman et al., 1994; Mohamed et al., 1999; He et al., 1999; Saleh and Connell, 1998). Differences between past data and our finding may be in part due to the different stimuli used; we used a psychological stressor where others have used pharmacological injections to induce changes in MAP.

To study the effects of estrogen treatment on MAP and HR responses to restraint, OVX-E and OVX-V animals were compared, but no differences were found. Similarly, when comparing the groups with little estrogen (OVX-V and males) no differences were found. Thus, this comparison suggests that





estrogen replacement elicits no changes in the cardiovascular response to stress. These results were unexpected as there is data suggesting that  $17\beta$ -estradiol replacement in postmenopausal women undergoing mental stress attenuates blood pressure response (Contreras and Parra, 2000).

When testing for differences due to ovariectomy and/or sexually dimorphic cardiovascular effects, large differences were found. Intact females exhibited approximately 20 % lower MAP than OVX-E and male rats, but no difference in HR. These results suggest that some peripheral effects on the vasculature may be responsible for the differences associated with endogenous ovarian hormones, rather than centrally mediated effects alone. These results can be supported by some previously published works where estrogen treatment of OVX rabbits enhanced endothelium-dependent relaxation of excised femoral arteries (Gisclard et al., 1988). In pharmacologically OVX rats given both estradiol and medroxyprogesterone acetate (frequently used in clinical hormone replacement therapy), the passive diameters of small arteries were significantly larger than those of OVX rats or those receiving medroxyprogesterone acetate alone (Acs et al., 2000). The suggestion that effects of restraint are in part peripheral are supported by the comparison between males and females, where females exhibited decreased MAP with no difference in HR measurements showing a sexually dimorphic response to restraint.

To describe the effects of estrogen replacement, ovariectomy and gender on autonomic responses to stress throughout this thesis, two types of



comparisons were made. First, comparisons of restrained groups to their unrestrained controls were done, and secondly, comparisons among restrained groups were done. The former comparison is more informative because it takes into account any changes in unrestrained animal responses. In other words, comparisons among restrained groups may miss significant changes within a group due to stress.

### **4.3. In situ hybridization for TH mRNA**

Data collected for TH mRNA analysis is somewhat incomplete due to technical problems, particularly for the control animal groups. Thus, any conclusions that may be drawn must be taken cautiously. With the data available, it is shown that there appeared to be higher levels of TH mRNA found in the LC of unrestrained, estrogen containing animals (OVX-E and diestrus) in comparison to the animals groups without estrogen. Therefore, it appears that estrogen may be associated with a higher level of TH mRNA in unrestrained animals. This finding appears contrary to previous data that estrogen attenuates sympathetic output in unstressed rats by lowering blood pressure (Hernandez et al., 2000), but we only tested for mRNA levels of TH and not protein levels, activity of the enzyme, or secretion of catecholamines.

Upon restraint, all four animal groups exhibit the same levels of TH mRNA in the LC. For this result to come about, the animal groups without estrogen show a significant increase in TH mRNA upon restraint whereas the groups with estrogen remain constant. Thus, it appears that the lack of



estrogen causes increased TH mRNA production upon restraint where the presence of estrogen causes the same level of TH mRNA in the presence or absence of restraint. These results confirm past findings that estrogen replacement attenuates the blood pressure response to mental stress (Sung et al., 1999) and attenuates sympathetic output when testing for the baroreflex (injections of nitroprusside or phenylalanine) (He et al., 1999; Saleh and Connell, 1998; Saleh and Connell, 2000). There is no significant effect of the ovariectomy, but there is a significant sexually dimorphic effect of restraint on TH mRNA. Some effects of the restraint stress may have been missed because our measurements were made after 4 hours of experimentation while other published data have shown that after 30 minutes of restraint stress in male rats TH mRNA increases by 40%, but after 2 hours it decreases back to baseline (Sands et al., 2000). Others have also found that 4 hours after hypotensive hemorrhage, TH mRNA levels return to control values (Chan and Sawchenko, 1995).

#### **4.4. NO production and NADPHd histochemistry in controls**

Diestrus rats had significantly lower nitrate/nitrite concentrations than male rats in the hypothalamus, and no other significant differences were found in the unrestrained control hypothalami or brain stems. Thus, a sexually dimorphic difference is seen in the NO production of unrestrained animals. The decreased NO production in females appears contradictory to known data



that unstressed OVX-E rats have higher plasma NO concentration than OVX rats (Hernandez et al., 2000). The differences seen between previously published data and our data may be associated with the type of NO being measured. In other words, previous works have measured plasma NO concentrations, whereas we measured NO concentrations in brain homogenates. Our results may also be linked to the presence of progesterone in intact diestrus females, and this hormone may be affecting the results.

One limitation of the NO assay we used is the inability to identify which neurons are responsible for the NO being produced. Thus, we used NADPHd staining to localize NO-producing neurons in the subdivisions of the PVN. When comparing numbers of positively NADPHd stained neurons in the PVN among the unrestrained controls, it was found that estrogen treatment lowered the number of NADPHd positive neurons in the magnocellular neurons (pm) and the ventral medial parvocellular (mpv) subdivision. These results differ from published data that find no increase in the number of NADPHd stained neurons upon estrogen treatment in the PVN of unrestrained guinea pigs (Warembourg et al., 1999). On the other hand, Warembourg et al. found that estrogen treatment increased the number of NADPHd stained neurons in the ventrolateral nucleus of unrestrained guinea pigs. The ovariectomy itself caused a decrease in NADPHd positive neurons in the pm, and a sexual dimorphic difference was seen in the mpv where females exhibited lower numbers than males. Since estrogen and/or ovariectomy effect the number of NADPHd positive neurons in the pm





subdivision during the basal state, they may have effects upon the production of hormones (OXY, AVP) in neurons that project to the posterior pituitary. Since it also appears that estrogen and/or gender may affect the number of NADPHd positive neurons in the mpv subdivision during the basal state, they may have effects upon CRF producing neurons that project to the anterior pituitary to effect sympathetic output. Before conclusions can be drawn using results from NADPHd staining, one must be aware of one limitation. NADPHd staining only indicates that neurons contain nNOS (Dawson et al., 1991; Hope et al., 1991), but the actual levels of NO production are not measured.

#### **4.5. NO production and NADPHd histochemistry in restrained rats**

When comparing the restrained rats to their controls, significant increases in NO production were seen in the hypothalamus associated with estrogen treatment and sex, and significant increases were seen in the brainstem associated with estrogen treatment and ovariectomy. When comparing NO concentration among the restrained groups, higher NO production in the hypothalamus and brain stem was seen associated with estrogen treatment and ovariectomy. Increases of central NO due to the estrogen replacement were hypothesized because data published previously show that estrogen replacement increases peripheral NO production (Lopez-Jaramillo et al., 1996; Best et al., 1998; Stefano et al., 2000; Weiner et al.,



1994). Increases due to ovariectomy may be due to the removal of endogenous ovarian hormones from the body, and this may cause a removal of NO inhibition. Little is currently known about the sexual dimorphic effect of restraint on central NO production.

Upon restraint, the 3 female groups exhibit higher numbers of positively NADPHd stained neurons throughout the PVN (the area responsible for HPA axis activity and sympathetic output) in comparison to controls. Significant increases in NADPHd positive neurons are only seen in the rostral PVN and the magnocellular neurons of male rats. Therefore, restraint may effect the HPA axis and sympathetic output via increased nNOS presence in middle and caudal PVN neurons of female rats. Perhaps testosterone and/or other androgens present in males can prevent the increase seen in females. Little is known about effects of estrogen treatment or gender on NADPHd staining in restrained animals.

Upon comparison among restrained groups, higher numbers of positively NADPHd stained neurons are seen associated with estrogen in the dp subdivision of the PVN (an area responsible for sympathetic output). This result may be related to the literature stating that estrogen causes increased resting plasma NO and decreased sympathetic output upon nitroprusside or phenylephrine injections (He et al., 1999; Saleh and Connell, 1998; Saleh and Connell, 2000; Lopez-Jaramillo et al., 1996; Best et al., 1998). We hypothesized that estrogen may be effecting sympathetic output and the HPA axis via NO production, but before conclusions about estrogen attenuation of



sympathetic output via NO can be drawn, experiments must be done to specifically show the association. A sample experiment could be the central injection of NOS blockers and measurement of the effects of estrogen treatment on HPA axis and sympathetic output.

Higher numbers of NADPHd stained neurons in the middle PVN subdivisions responsible for both HPA axis activity (mpd) and sympathetic output (dp and mpv) are seen in restrained animals due to ovariectomy. Therefore, removal of endogenous ovarian hormones (other than estrogen) may affect the number of NO-producing neurons in the PVN, and thus effect the HPA axis and sympathetic output.

#### **4.6. ACTH and CRF mRNA controls**

No differences in ACTH plasma concentration were seen among the unrestrained groups suggesting that all the groups of rats have similar basal HPA axis activity.

There was a trend for all 3 unrestrained female groups to have higher CRF mRNA levels than males in the subdivisions of the middle PVN responsible for sympathetic output and HPA axis activity, where levels in OVX-V and diestrus rats were significantly higher in the dp and mpd. Thus, there is a sexually dimorphic difference in CRF mRNA levels in the PVN of unrestrained animals. Since unrestrained males showed less CRF mRNA than the females, the presence of testosterone and other male androgens may be



associated with this decrease, and thus associated with the potentially attenuated HPA axis activity. Previous data have also concluded that testosterone normally acts to inhibit the HPA axis response to perturbations (Reviewed in Handa et al., 1994). In order to test this, we could measure the levels of CRF mRNA in castrated male rats. Whereas results in this thesis find no effect of ovariectomy or estrogen treatment on CRF mRNA levels, past data suggest that both inhibit CRF immunoreactivity in the median eminence of unstressed rats (Haas and George, 1989). Further studies have shown both estrogen and progesterone to effect basal levels of CRF mRNA. Estrogen was shown to increase CRF mRNA signal in the resting monkey PVN, and animals also given progesterone exhibit attenuated CRF mRNA levels not different from animals with OVX alone (Roy et al., 1999). Little data is available for the effects of estrogen treatment, ovariectomy, or gender on the levels of plasma ACTH or CRF mRNA levels in the PVN in unstressed animals, but it appears that all three are involved.

#### **4.7. ACTH and CRF mRNA in restrained rats**

We measured ACTH levels at the end of the 4 hour restraint/rest protocol. Therefore, results about the effects of estrogen on ACTH release in response to restraint may have been missed due to two things. First of all, there are data suggesting that differences in ACTH plasma concentrations are usually seen within an hour of initial stress (Burgess and Handa, 1992). Since





the half-life of plasma ACTH in both human and rat is less than 1 hour (Lu et al., 1983; Lopez and Negro-Vilar, 1988), ACTH measurements at 15-minute intervals immediately after the application of restraint are currently underway in our lab. Secondly, the secretory rates of CRF, ACTH, and cortisol vary throughout the day (high early in the morning and low in the late evening)(Chapter 77 in Guyton, 1991), and the plasma recovery times used in this thesis vary by up to 5 hours.

When comparing the restrained animal groups to the corresponding unrestrained controls, effects are seen associated with estrogen treatment and gender. Estrogen appears to keep ACTH production constant upon stress, and vehicle treated animals show significantly increased ACTH production upon restraint. Males exhibit decreased ACTH production upon stress compared to females, and since there are no gender-dependent differences in the half-life of human ACTH (Roelfsema et al., 1993), differences may be related to restraint. Since the ACTH concentration does not change in the animals with estrogen, perhaps estrogen causes a stabilization of the HPA axis during stress. Previous data have opposite results to ours showing increased ACTH production in both intact males and randomly cycling females due to exposure to novel stressors with higher ACTH production in females than in males (Handa et al., 1994). Differences may again be associated with time of plasma recovery. Handa et al. recovered the ACTH plasma samples 20 minutes after initial stressor, whereas we collected ACTH plasma samples 4-hours after initial stressor.



In contrast to the comparison of unrestrained animals, restrained male animals exhibited a significantly lower ACTH plasma concentration than all of the restrained female groups. Thus, it appears that upon restraint stress, the females may have higher HPA axis activity than do males. Again it appears that the presence of testosterone and other androgens may contribute to decreased HPA axis activity, but in this case the results are due to restraint stress and not just basal conditions. If testosterone were a factor, it could be tested by analysis of ACTH plasma concentration of castrated male rats. Again, data have shown in the past that testosterone decreases the HPA axis response to stress, where castrated males exhibited increased ACTH production (Reviewed in Handa et al., 1994).

Where we found no differences in ACTH production among the restrained female groups, it has previously been found that after footshock stress, plasma ACTH concentration of OVX-E rats was significantly higher than OVX rats. These results indicated augmentation of the HPA axis upon estrogen treatment (Burgess and Handa, 1992). These differences may be due to the time allowed from initial stress. Burgess and Handa measured ACTH throughout 2 hours after initial stress, where we measured ACTH concentrations after 4 hours.

When comparing the stressed groups to the corresponding unrestrained controls, there were no significant differences in CRF mRNA levels associated with estrogen treatment. There appeared to be increases related to the ovariectomy and opposing effects on CRF mRNA levels due to gender in



the dp, mpv and mpd subdivisions (areas responsible for regulation of sympathetic output and HPA axis activity). In other words, attenuated CRF mRNA in females is shown upon restraint and augmented CRF mRNA in males is shown upon restraint. These results are likely due to endogenous female and male hormones. This difference could also be related to previously published data that after 1 hour of restraint stress glucocorticoid (GC) receptor mRNA is upregulated in the male hypothalamus while down regulated in the female hypothalamus (Karandrea et al., 2000). There is some evidence that prolonged estrogen treatment of old male rats increases GC receptor immunoreactivity in the hippocampus, thereby enhancing the feedback signal (Ferrini et al., 1999). Therefore it appears that progesterone may play a role in down-regulating GC receptors as well. As mentioned previously, another experiment that could be done is to test the levels of CRF mRNA in castrated male animals to analyze the effects of testosterone.

Many of the CRF observations lead to potential contradictions of the conclusions from the ACTH data. There are no significant differences in CRF mRNA levels seen in the PVN among stressed groups, whereas there was significantly lower plasma ACTH concentration in the male rats. This might be explained by a possible difference in the translation of CRF mRNA leading to differing amounts of CRF hormone, or a different secretion rate of the hormone to the anterior pituitary. These results may also be due to the immediate release of ACTH from the anterior pituitary, while synthesis of CRF mRNA requires more time.



#### 4.8. Long-term Measurements

Estrogen replacement therapy in humans is used for prolonged periods of time. To measure the effect of long-term estrogen replacement on OVX female rats we measured the effects of 7-week estrogen treatment on the cardiovascular and HPA axis response to restraint in rats. Data collected for long-term experiments is preliminary and all experiments done are in restrained animals only. LTE rats had lower blood pressure and lower heart rate compared to LTV rats. These results indicate decreased sympathetic output in LTE rats. There is no ACTH difference between LTV animals and LTE animals. NO production in the hypothalamus of LTE animals is significantly lower than LTV animals, but is significantly higher in the brainstem. When comparing LTE and LTV restrained animals, a significant increase in the number of NADPHd stained neurons was seen in the magnocellular neurons of the LTE rats.

There are many differences found between autonomic responses of short-term treated rats and responses of long-term treated rats to restraint. Where there were no differences in MAP or HR in short-term treated rats, it appears that long-term estrogen treated animals have attenuated sympathetic output. In contrast to the observations made from short-term treated animals where estrogen treatment increased NO production in the hypothalamus, long-term estrogen treatment shows significantly lower NO production in the





hypothalamus. The significant increase in the number of NAPHd stained neurons in the dp seen in short-term estrogen treated restrained rats was not present in long-term estrogen treated restrained animals, but a significant increase in the number of NADPHd stained neurons was seen in the magnocellular neurons of the long-term treated restrained rats. Since the number of nNOS containing neurons changes, there may be a potential difference in the regulation of these areas. For example, since increased numbers of nNOS containing neurons are seen in the magnocellular neurons of long-term treated rats, perhaps regulation of this subdivision has changed.

Two possible reasons exist for the differences seen between short-term treated animals and long term treated animals. First of all, an important thing to note is that LTE treated animals had a plasma estrogen concentration of only  $28.15 \pm 4.3$  pg/ml where the OVX-E animals had a plasma estrogen concentration of  $66.2 \pm 14.2$  pg/ml. Animals were each given an estrogen replacement pellet, where LTE rats received a 60 day release pellet and OVX-E rats received a 21 day release pellet. The delivery dosage resulted in the LTE rats exhibiting 1/3 the estrogen plasma concentration of OVX-E rats. Since significant attenuation of cardiovascular responses to stress occurred in animals receiving less estrogen, this indicates that estrogen replacement is beneficial even in smaller concentrations. Secondly, long-term treatment, or the difference in estrogen concentration, may affect the numbers of estrogen receptors (ER) and/or glucocorticoid receptors (GR) in the brain. For example, six-week estrogen treatment of old male rats caused increased GR in the



hippocampus following ether stress (Ferrini et al., 1999), and two weeks of estrogen treatment changes the level of ER subtypes throughout the brain (Osterlund et al., 1998). Thus, it has been shown that extended estrogen treatment can effect the level of receptors, which can then elicit potentially different effects on HPA axis and sympathetic output than short-term estrogen treatment.

Weight gain among all 4 groups was significantly different after 7 weeks. Males were the heaviest followed by LTV, intact females, and finally by LTE animals. From this data one can see that even though the plasma estrogen concentration found in LTV and LTE is not significantly different, there is a significant difference in weight gain. Thus, there must be an effect of the estrogen replacement on weight gain. There are data showing that OVX rats exhibit higher weight gain than cycling rats in diestrus (Davidge et al., 2001) which agrees with our findings. A sexually dimorphic effect of weight gain has been shown where male rats gain significantly more weight than intact cycling females over a 7 week period. Thus, there must be some effects of male and/or female endogenous hormones on weight gain. Past data have shown that castration of adult male rats results in a reduction in weight gain, and injections of testosterone propionate (aromatizable androgen) stimulate weight gain (Gentry and Wade, 1976).

The final step in estrogen biosynthesis is the aromatization of androgens via an enzyme called aromatase. Aromatase is found in gonads, adipose tissue, and the brain (Reviewed in Rissman et al., 1999). Thus,



animals with a higher amount of adipose tissue have more aromatase, and thus have increased ability to convert androgens into estrogen. OVX rats given exogenous aromatase show significantly less weight gain than diestrus rats, indicating that estrogen can inhibit weight gain (Davidge et al., 2001). These data again support our findings. Perhaps the long-term treated animals had exaggerated responses to stress due to estrogen synthesized via aromatase in their increased levels of adipose tissue. Furthermore, since aromatase is found directly in the brain, estrogen can be synthesized there and elicit central responses more quickly than via the circulation.

#### **4.9. Summary and Conclusions**

The autonomic nervous system responds to stress via centrally mediated pathways; two important pathways discussed are sympathetic output and the HPA axis. Measurements of these two pathways, and central NO production during and/or after restraint stress show influences of estrogen treatment, ovariectomy, and gender. We have shown that estrogen treatment 1) attenuates cardiovascular responses to stress via more efficient MAP and HR responses, 2) significantly increases central NO production after restraint, and 3) attenuates HPA axis response to restraint in comparison to vehicle treated rats. These results might explain the reduced risk of cardiovascular disease seen in postmenopausal women taking estrogen replacement therapy compared to postmenopausal women without estrogen replacement



(Reviewed in Mendehlsohn and Karas, 1999). We have also shown that the ovariectomy 1) attenuates the baroreflex, 2) augments central NO production after restraint, and 3) augments HPA axis response (CRF mRNA) to restraint. Finally, we have shown a sexually dimorphic effect of restraint. In comparison with intact males, females exhibit 1) more efficient baroreceptor reflex upon restraint, 2) significantly increased central NO production upon restraint, and 3) attenuated HPA axis response (CRF mRNA) to restraint.





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